UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine, Healthcare and Life Sciences

School of Medicine

THE ENHANCEMENT AND ENRICHMENT OF SKELETAL STEM CELLS AND IMPACTION BONE GRAFT FOR ORTHOPAEDIC APPLICATION

Andrew M H Jones

Thesis for the degree of Doctor of Medicine,

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<u>**Abstract**</u>

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With an ageing population putting ever increasing demands on the musculoskeletal system there is a growing need for the development of regenerative medical strategies to provide for the healthcare needs of the future. With increasing numbers of joint arthroplasty occurring in younger and younger patients there is likely to be a growing need for therapeutic strategies to replace lost bone stock in the coming decades

This thesis aims to explore strategies to enhance the biological and mechanical properties of impaction bone grafting and the effects of skeletal stem cell (SSC) concentration. In order for SSC to be effective in replacing new bone stock new strategies looking to enhance osteogenic differentiation have been examined.

Section I: An *in vitro* Impaction Bone Graft (IBG) model of SSC seeded onto human allograft was used to study the biomechanical effects of altering SSC concentration. The use of concentrated SSC was then used in the treatment of patients with avascular necrosis (AVN) of the femoral head and fracture non union with parallel in vitro analysis of the samples.

Section II: In vitro and murine *in vivo* analysis of the biomechanical effects of type 1 Collagen and Hydroxyapatite nanoparticles primarily looking at differences in shear strength and osteogenic differentiation compared to plain allograft and basal cultured SSC.

Section III: An *in vitro* acetabular model was used to study the effect of vibration IBG compared to standard techniques in revision hip surgery. The graft compaction, force of impaction, fracture risk and rate of subsidence post cyclical loading was assessed.

This thesis has demonstrated *in vitro* and *in vivo* strategies that are clinically translatable and have demonstrated that:

- Skeletal Stem Cell concentration plays a pivotal role in the biomechanical enhancement of Impacted bone graft (IBG)
- Translation of these strategies into the successful treatment of fracture non union and Avascular necrosis of the hip.
- Type 1 Collagen and Hydroxyapatite nanoparticles both enhance the osteogenic differentiation and shear strength of the IBG / SSC construct
- Vibration impaction bone grafting is a novel technique that significantly reduces the intraoperative risk of acetabular fracture or catastrophic subsidence

This thesis has demonstrated novel techniques for the biomechanical enhancement of IBG with most techniques being readily transferable to clinical practice with the potential to form part of a surgeon's armament for regenerative medical techniques of the future.

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Academic Thesis: Declaration Of Authorship

I, Andrew M H Jones declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

THE ENHANCEMENT AND ENRICHMENT OF SKELETAL STEM CELLS AND IMPACTION BONE GRAFT FOR ORTHOPAEDIC APPLICATION

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at the University of Southampton

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

7. Parts of this work have been published before as documented in the publications section;

Signed: ....

Date: ....

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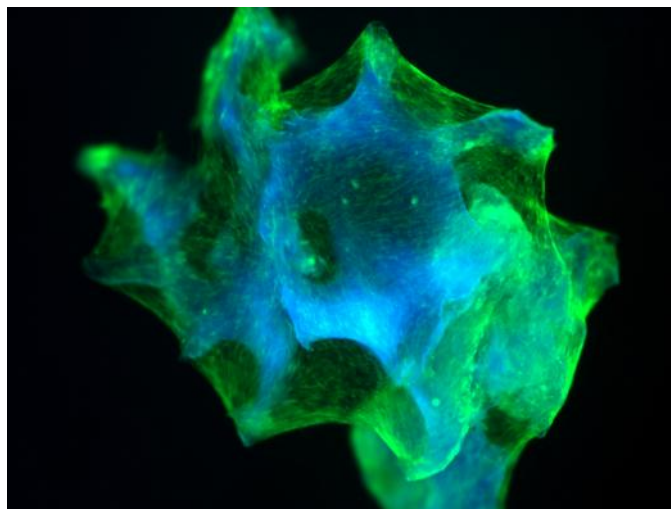
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Publications + Abstracts

Skeletal Stem Cells And Bone Regeneration – Translational Strategies From Bench To Clinic

Rahul S Tare; Janos M Kanczler; Alex Aarvold; Andrew Jones; Douglas G Dunlop;
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Biomechanical Enhancement Of Impaction Bone Grafting

Mr A.M.H.Jones, Miss T.S.Foong, Dr AM New, Mr B.J.R.F Bolland, Dr J.C.Pound, Mr D.G.Dunlop, Prof R.O.C. Oreffo.

From Roadside To Revision Hip Surgery: The Role Of Vibration Impaction In Acetabular Impaction Bone Grafting

AMH Jones, AM New, BJRF Bolland, ROC Oreffo, DG Dunlop

The Effect Of Skeletal Stem Cells, Hydroxyapatite Coated Stem Cells And Collagen Coated Allograft On The Biomechanical Properties Of Impacted Bone Graft

Mr A.M.H.Jones, Miss T.S.Foong, Dr AM New, Mr B.J.R.F Bolland, Dr J.C.Pound, Mr D.G.Dunlop, Prof R.O.C. Oreffo.

Enhancing The Properties Of Impacted Bone Graft By Coating With Collagen And Skeletal Stem Cells

AMH.Jones, TSFoong, AM New, BJRF Bolland, DG Dunlop, ROC Oreffo

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Presentations

Enrichment of Skeletal Stem Cells From Bone Marrow to Enhance Skeletal Regeneration – A Novel Clinical Technique

J.O.Smith, JI Dawson, A Aarvold, AMH Jones, JN Ridgway, SJ Curran, DG Dunlop, ROC Oreffo.

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A Novel Approach To Improve Impaction Bone Grafting In

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Poster presentation, EFORT June 2009

Use Of Skeletal Stem Cells And Impaction Bone Grafting In The Treatement Of Avascular Necrosis.

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Podium presentation at the Tissue and Cell Engineering Society Annual Conference

Acetabular Vibration Impaction

A.M.H. Jones, B.J.R.F. Bolland, A.M. New, R.O.C. Oreffo, D.G. Dunlop

Oral Presentation, Exeter Hip Development Meeting Exeter Feb 2008

From Roadside To Revision Hip Surgery: The Role Of Vibration Impaction In Acetabular Impaction Bone Grafting

AMH Jones, AM New, BJRF Bolland, ROC Oreffo, DG Dunlop

Poster presentation, British Hip Society Meeting, Manchester 2009

Biomechanical Enhancement Of Impaction Bone Grafting

Mr A.M.H.Jones, Miss T.S.Foong, Dr AM New, Mr B.J.R.F Bolland, Dr J.C.Pound, Mr D.G.Dunlop, Prof R.O.C. Oreffo.

Poster Presentation, Bone Research Society Meeting UCL June 2009

Other Media Publications

BBC Health - 6/04/2008 "Bone repairing stem cell jab hope"

SKY News – 31/08/2009 "Stem Cells Op Could End Hip Replacements"

Telegraph – 1/09/2009 "New stem cell treatment being used by patients to avoid hip replacements"

Prizes

The Engineer Awards 2010	Medical /Healthcare section winner
The Engineer Awards 2010	Grand Prix Special Award for overall winner
Top 10 of 2010	Awarded top medical device of 2010. Orthopaedic Stem cell concentrator developed in conjunction with Smith & Nephew
First Prize Presentation	Postgraduate Research Conference, Southampton 2011
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Prize Oral Presentation	Gauvain Society Annual Conference 2008
'Best of the Best' Abstract	Selected for the Best of the Best section, BOA 2009

Abbreviations

ALP	Alkaline phosphatase
ASC	Adipose Derived Stem Cells
AVN	Avascular necrosis
A/S	Alcian blue / Sirius red
BMA	Bone marrow aspirate
c	*circa*
CFU-F	Colony forming unit - fibroblast
CTG-EH	CellTracker Green – Ethidium Homodimer
DNA	Deoxyribonucleic acid
ECM	Extra-cellular matrix
FCS	Foetal calf serum
HAp	Hydroxyapatite nanoparticle
HBMSC	Human Bone Marrow Stromal Cell
HFN	Human Fibronectin
IBG	Impaction Bone Grafting
IL	Interleukin
µCT	micro Computed Tomography
MCP-1	Monocyte chemotactic protein - 1
M-CSF	Macrophage colony stimulating factor
MEM	Modified Eagle's medium
MIP-1	Macrophage inflammatory protein - 1
OPG	Osteoprotegerin
RANKL	Receptor Activated by NF B Ligand
RGD	Arginyl-Glycyl-Aspartic acid
SEM	Scanning electron microscopy
SSC	Skeletal stem cell
TCP	Tricalcium Phosphate
TGF-	Transforming growth factor -
THR	Total hip replacement

Major Null Hypothesis

Novel new surgical techniques and Increasing skeletal stem cell concentration and osteogenic differentiation by manipulation with extracellular membrane proteins and apatite crystals has no effect on the biomechanical properties of Impaction Bone Graft

The study has been divided into three main sub sections:

Section I – Biomechanical effects of SSC concentration in IBG
- In vitro study
- Clinical translation and parallel in vitro assessment

Section II – Enhancing Osteogenic Differentiation of SSC
- In vitro analysis of Type 1 Collagen
- In vitro analysis of Hydroxyapatite nanoparticles
- In vivo murine study

Section III – Novel Impaction bone grafting technique
- In vitro mechanical study

Chapter 1

Introduction

1:1 Burden of the Problem

The requirement to reconstruct bone defects presents an increasing burden to healthcare in the face of the demographic challenges of an increasing aged population. The proportion of people in Britain aged over 65 years is estimated to increase by 50% in the next 25 years (ONS 2008). A concomitant increase in surgery for orthopaedic complications can be expected, which emphasizes the need for innovative approaches to skeletal reconstruction.

While traditional causes of bone loss such as trauma, infection and tumours present ongoing challenges, there is now a growing cohort of patients who require bone defect reconstruction during revision arthroplasty surgery. In 2008 in England and Wales, there were approximately 65,000 primary hip replacements performed in comparison to just 30,000 in 2003, and the number of revision hip procedures doubled over this period, to 6,600 (NJR 2009). Given these demographic changes and that joint replacements are being increasingly performed in younger patients, the number of people outliving their prosthesis can be projected to increase. Patients now present in rapidly increasing numbers, often with extensive bone loss around loose implants, which poses a substantial orthopaedic challenge for revision surgery. In addition, knee arthroplasty is now performed more frequently than hip

arthroplasty (NJR 2009), thus the true scale of the problem of inadequate bone stock in revising total knee replacements will only emerge in years to come.
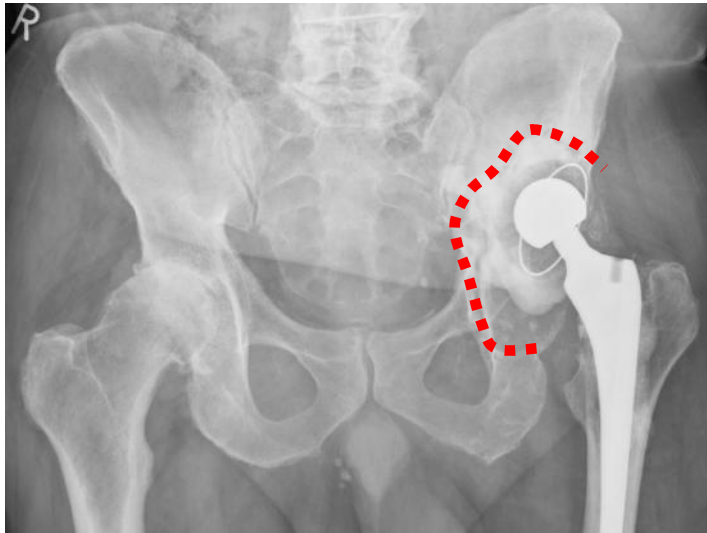
Fig 1:1 Radiograph of a failing left total hip replacement. Dashed line signifying the extent of acetabular bone loss with a large cavity formed by the loose component. (Courtesy of the Royal Berkshire Hospital)

Furthermore, the patient of today has higher expectations of medical intervention (Nilsdotter, Toksvig-Larsen et al. 2009) and a deteriorating, painful prosthetic joint will often be poorly tolerated in the context of bone loss occurring as a result of a previous therapeutic intervention.

1:2 Impaction Bone Grafting

Impaction bone grafting (IBG) using fresh frozen morselised allograft remains the current gold standard for replacing bone stock encountered during revision hip surgery and has been used as a void filler as such for forty years (Slooff, Huiskes et al. 1984). The technique, which employs fresh frozen morselised allograft as a passive 'void filler', endeavours to recreate a solid, adaptive

mantle into which the implant can sit securely, immediately withstanding forces associated with normal implant use.

IBG for the acetabulum was first introduced by Slooff et al in Nijmegen in the late 1970s (Slooff, Huiskes et al. 1984) with Ling and the Exeter group describing the technique for Femoral IBG (Ling, Gie et al 1993). The technique for acetabular IBG is shown in Fig 1:1.
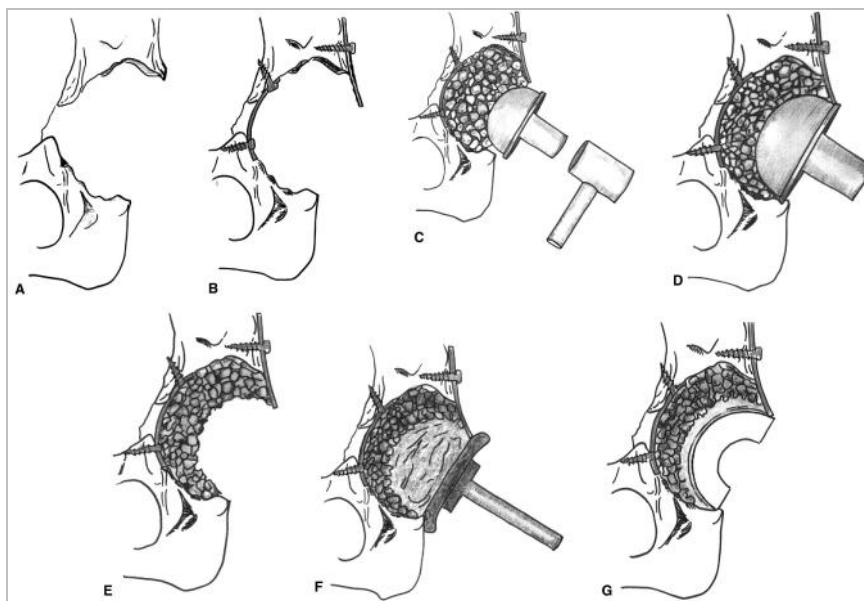
Fig 1:2 Schematic diagram showing the key steps in IBG of the acetabulum. A: Acetabular defect with cavities. B: Bridging of defects with mesh. C,D: Impaction of Bone Graft chips with tamp. E: Final impacted bed. F: Cement pressurisation into bed. G: Implantation of Acetabular cup into cement base. Adapted from Schreurs BW, Gardeniers JW, Slooff TJ: Acetabular reconstruction with bone impaction grafting: 20 years of experience. *Instr Course Lect* 2001;50:221-228.

It involves firstly creating a contained defect, for which a metal mesh or large allograft blocks can be used to cover any defects in the cavity wall. Multiple superficial drill holes can be made in the bone to help initiate vascular invasion at this stage. The graft bed is then built up in a step wise fashion with small

amounts of allograft added and impacted before adding further graft. Once the cavitatory defect has been filled sufficiently with compacted allograft cement is then pressurised into the cavity in a relatively viscous state and an appropriate cup (2-4mm smaller than the last tamp diameter) is implanted. (Adapted from Exeter Xchange revision acetabular surgical protocol)

Survivorship for acetabular and femoral components at 20 and 10 years respectively is encouraging (87% and 99%) although such results have not always been repeated outside specialist centres (Eldridge, Smith et al. 1997; van Haaren, Heyligers et al. 2007). van Haaren and colleagues from Nijmegen reviewed 71 Cases of Acetabular revisions with IBG and found a survival rate of 72% (95% CI, 54.4 to 80.5) at 7 years for aseptic loosening. The majority of failures occurring in those with severe defects with poor graft incorporation being the main cause (van Haaren, Heyligers et al. 2007). A recent review of 61 cases by Schreurs et al has shown an overall survival rate at 20 years of 79% although once revision for other causes are excluded, the revision rate for asceptic loosening is 87% (Schreurs et al 2009). So whilst the revision rate for what can be significant bone loss is encouraging, there is clearly potential to improve this technique and the survivorship further.

The ability of the graft bed to resist the forces placed upon it and form a new living bony construct is a critical factor for the long-term success of the revision implant. However, histological specimens from acetabular IBG reveal fibrous and necrotic tissue amongst areas of graft integration (van der Donk, Buma et al. 2002; van Haaren, Heyligers et al. 2007). The process of replacing and

remodelling of the IBG bed occurs by creeping substitution (Burchardt, Hans 1983) and therefore as the depth of bed increases so the risk of central necrotic areas increases as the distance from the host blood supply increases. Weidenheilm et al (Weidenheilm L.R et al 2001) performed histological analysis of retrieved IBG samples and described three main zones of the graft bed. There was a central / inner zone that contained both the bone cement but also areas of necrotic graft and trabeculae along with some vascularised fiborous tissue. The middle zone contained viable trabecular bone with an incomplete peripheral cortex. The final outer zone was made up of viable cortex. Whilst this composite histological structure of the IBG does appear to offer good structural support for the majority of cases, there is clearly potential for the necrotic areas to collapse. The majority of cases of failure in van Haaren et al's study were those with severe defects, and it may be the case that once the graft bed exceeds a certain depth, tissue necrosis in the 'inner' zone becomes inevitable (van Haaren, Heyligers et al. 2007).

The application of tissue engineering strategies to promote the generation of host bone, and thereby improve the biomechanical characteristics of the graft, has potential to improve the outcomes of this demanding technique. Recent work within our laboratory, and also by others, has identified mechanical and biological factors that can be modulated to augment the structure and performance of IBG (Shetty, Slack et al. 2005; Bolland, Partridge et al. 2006; Bolland, Tilley et al. 2007). Factors, such as preparation of graft, constituent particle size, cellular composition, chemical additions and local fluid drainage have all been shown to affect the performance of IBG (Dunlop, Brewster et al.

2003; Bolland, New et al. 2008; Jones AMH et al 2009). Whilst skeletal stem cells (SSCs) in themselves have been shown to augment the biological and mechanical properties of impaction bone grafting (Bolland, Partridge et al. 2006).

1:3 Allograft

Allograft is usually obtained from consenting patients undergoing primary total hip replacement but also from consented cadaveric retrieval. The different techniques of processing and storage of the allograft prior to use has been studied extensively. The main three options being fresh frozen, freeze dried and irradiated allograft. All have advantages and disadvantages with a compromise between prevention of potential disease transmission (Simonds et al., 1992; Lord et al., 1988; Buck et al., 1989), immunogenic response (Eldridge et al., 1997) and disruption of the mechanical and osteoinductive properties of the graft (Schreurs et al 2004). Irradiation of the graft, whilst reducing the risk of disease transmission has been shown to have inferior mechanical properties with reduced graft incorporation (Delloye C et al 2007; Hannink G et al 2007) although this is debated and there is evidence to the contrary (Hannik G et al 2007). In a clinical study on acetabular IBG Ochs BG et al showed that the results of combining irradiated graft and Bone marrow aspirate were comparable to plain fresh frozen allograft at 3 years follow up (Ochs et al 2010). Their study of 79 acetabular reconstructions showed comparable Harris hip scores at final follow up, and 100% incorporation of the graft beds as demonstrated by trabeculation on plain radiographs. There were

no signs of acetabular loosening at final follow up, although it should be noted that all reconstructions were with the use of a reinforcement ring in addition to the IBG and therefore the mechanical demands of the IBG construct were not as great as an IBG alone.

Fresh frozen allograft has the advantage of retaining some of the osteoinductive properties but does carry increased risk of an immunogenic response. In order to reduce this response the mechanical effects of pulse lavage washing and de-fatting of the samples prior to IBG has been studied (Dunlop DG, Brewster et al 2003). This demonstrated that there was a significant mechanical advantage to washing of the allograft with further benefit from de-fatting with Hydrogen Peroxide.

The grading of the allograft particles has also been shown to be a critical factor (Dunlop et al 2003). The key of any impacted aggregate is to allow a tight inter-digitation between the particles to prevent any movement under shear. In order for this to be optimised the aggregate needs to be 'well graded' i.e it is beneficial to be made up of particles of multiple different sizes. This allows the larger particles to form the main basis of the aggregate to provide structural support, but the smaller particles can fill the voids and lead to a more densely packed aggregate (Smith GN 1990) See Fig 1:2.

Calculation of the shear strength of an aggregate such as IBG is done using the Mohr Coulumb Failure Law, the process of which is discussed in more detail in section 2:5.

Fig 1:3 Schematic representation of impacted allograft particles demonstrating the importance of multiple particle sizes to allow dense packing of the graft and filling of any voids

The optimal graft characteristics would include Osteoconductive (the ability to provide structure for the attachment and growth of cells), Osteoinductive (contain active factors to stimulate cell migration and differentiation) and Osteogenic (contain cells from the osteogenic lineage)_properties. Fresh autollogous graft has all three of these properties, in the context of IBG, the volumes of graft required are not available and would cause significant morbidity. Fresh Frozen Allograft has been shown to have osteoconductive and some osteinductive properties due to the retained osteoinductive agents (Bormann et al 2010). The osteoinductive effect is however very small and this is further diminished by repeated washing of the allograft (Dunlop 2003).. For new bone formation another critical factor is that of vascular in growth or angiogenesis. In order for these new vessels to form and survive requires a

high level of stability within the graft bed as any shear can disrupt their formation remodelling (Hernigou et al. 2005 ;Stevenson et al 1996).

Other factors affecting the use of allograft are the supply and storage of the graft. Introduction of the Human Tissue act in the UK in 1984 has ensured traceability and control of the storage of all allograft samples used, to allow any tracking of potential disease transmission. The storage of the allograft is also required to be in a licensed facility which has meant the majority of UK Hospitals no longer have an in-house 'bone bank' with femoral heads having to be ordered in for individual cases. This has led to significant logistical issues for hospitals wishing to use it which puts further burden on a stretched NHS service. There has also been concern raised that the potential demand for allograft could outstrip supply given the predicted rise in revision arthroplasty (Delloye et al 2007). Further work has therefore shifted to look at potential synthetic scaffolds that can act as either supplements or replacements for fresh frozen allograft.

1:4 Bone Graft Extenders

To counter some of the issues described above regarding allograft a variety of synthetic scaffolds have been proposed and studies both as additives to the allograft and on their own. Early studies (Brewster et al 1999) looked at the effects of addition of an inert bioglass compound and showed that these increased the shear strength of the IBG construct, possibly due to improving the grading (mix of particle sizes) of the compound. Hydroxyapatite and tricalcium phosphate (TCP) ceramics have also been used as extenders and have shown osseointegration (Ransford A et al 1998). There have however

been some concerns raised about their ability to maintain a stable structure under loads (Hanft J et al 1995).

'Bonesave' (Stryker, Howmedica) a compound made of 80% TCP and 20% HA has been shown to be an effective bone graft extender with good results in a sheep model at up to 90% extender : 10% Allograft mix in a sheep IBG model (Blom AW et al 2005) with similar fusion rates found in a clinical study on spinal fusion using Bonesave (Kapur RA et al 2010) .

Fig 1:4 Bonesave granules used as bonegraft extender. (Stryker Howmedica)

Polymeric scaffolds have attracted interest in the field of tissue engineering due to their inert nature, and ability to modify the structural shape comparatively easily. An example of these scaffolds are Polylactic acid (PLA) and Polyglycolic acid (PLG) which are manufactured using high pressure carbon dioxide that can be modulated to affect porosity and composition (Kanczler et al 2008). These have been shown to offer similar mechanical properties under shear, but are also biocompatible and support the proliferation of HBMSC (Bolland et al 2008). A further advantage of some of the bone graft extenders is the ability to incorporate other factors such as growth factors into the structure to change them from merely osteoconductive to osteoinductive and to control SSC differentiation and potential angiogenesis (Kanczler et al 2008 & 2010).

1:5 Bone Graft additives

The process of Bone formation and remodelling is a highly complicated synergistic process involving the fine balance of Osteoclastic resorption and Osteoblastic bone formation. New bone formation is dependant upon an adequate blood supply to deliver nutrients, oxygen and precursor cells to the tissue, and so the use of factors controlling the formation of new blood vessels may be useful. Kanczler et al has shown that the use of Vascular Endothelial Growth Factor (VEGF) can be incorporated into scaffolds and cause enhanced vessel and new bone formation (Kanczler et al 2010). Bone Morphogenic Proteins (BMP) have also been used to try and stimulate new bone formation. They are multifactorial growth factors that belong in the Transforming Growth Factor (TGF-) superfamily. The activity of BMP was first identified in the 1960s (Urist et al 1965) with the proteins responsible for bone formation described by Wozney et al 1988 amongst others. BMP-7 (also known as osteogenic protein-1 / OP-1) and BMP-2 both play a key role in the differentiation of SSC into bone and cartilage and have been used in a number of studies and clinical applications (Ristiniemi et al 2007; .Vaidya R et al 2007). Their function is not purely on enhancing osteogenic differentiation however, and there is also stimulation of Osteoclast activity which in some studies may account for problems of subsidence due to increased osteoclast led resorption (McGee et al 2004). McGee's study was a sheep IBG hemiarthroplasty model with 6 control plain IBG and 6 sheep with an IBG OP-1 mix (OP-1 concentration ranged from 60 to 89 pg OP-I per 1g of bone). Analysis of the samples was carried out at 6, 18 and 26 weeks with 2 from each group sacrificed at each time point. Mc Gee reported improved graft incorporation

and remodelling in the OP-1 group, but with that, evidence of accelerated bone resorption compared to plain allograft with corresponding femoral stem subsidence in one of the OP-1 group at six weeks. These results should be interpreted with caution however as it is only a small pilot study and further work on the optimal dosing of OP-1 in the IBG setting is required.

More recently the use of Platelet Rich Plasma (PRP) has been gaining a lot of media attention as a potential cure for joint and tissue damage. As yet there are no large scale clinical trials, although results from some studies are encouraging, there remains significant scepticism as to the true benefit (Alsousou et al 2009; Moran et al 2010). The underlying rationale for its use is that it contains a multitude of concentrated growth factors such as VEGF, TGF-, Platelet Derived Growth Factor and Interleukins which can in theory all work in a synergistic fashion to induce tissue repair.

1:6 Skeletal Stem Cells

A stem cell is a cell that has the ability to self renew, differentiate into a number of different cell types and to maintain the ability to divide and replicate throughout its life. A wide variety of names have been given to the cell which for the purpose of this study will be termed Skeletal Stem Cell (SSC) (Bianco and Robey 2001). Other names given have been bone marrow stromal cells, Mesenchymal stem cells (Goshima et al. 1991) and marrow stromal fibroblastic cells (Triffitt et al 2002).

Most Stem cells can be isolated by their cell surface markers, but whilst the surface markers for most haemopoetic stem cells are clearly defined, the specific surface markers for SSC are not. There are a number of markers that can be used to narrow down the SSC yield of a sample, but none are exclusive or specific to SSC alone (e.g they are negative for some haemopoetic markers CD34, CD45, CD14, and positive for Stro-1, CD29, CD 73, CD90, CD105, CD166 and CD44) (Dominici et al 2006; Abdallah et al 2005; Foster et al 2005). The most commonly used antibody for SSC isolation is the STRO-1 antibody for a trypsin resistant surface marker found on most cells within CFU-F colonies (Simmons et al 1991; Deschaseaux et al 2003; Stewart et al 2003). Prospective isolation of true SSC can therefore be problematic, traditionally their propensity to adhere to tissue culture plastic compared to haemopoetic cells can be used to isolate by monolayer culture however analysis of the CFU-F cells has shown that only around 30% are multipotent and therefore true SSC (Grothos et al 2003).

Multipotent skeletal stem cells (SSCs) present in bone marrow, have the potential to differentiate down a number of different cell lineages including osteoblasts, chondrocytes, fibroblasts and adipocytes (Fig 1:2).
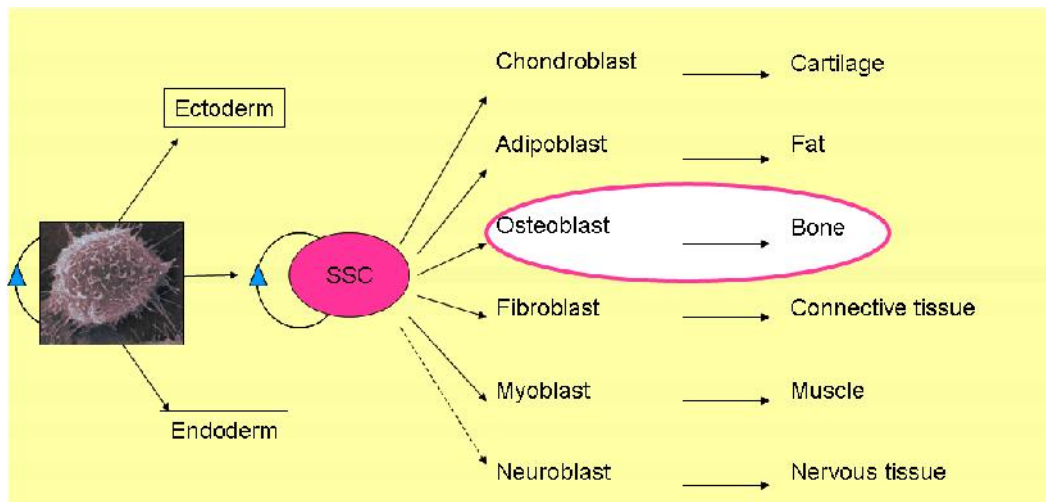
Fig 1:4 Schematic diagram showing the mesenchymal lineage with differentiation of SSC to their differentiated progeny. (Adapted from Oreffo, Cooper et al 2005)

In IBG it is desirable to maximise the differentiation of these stem cells to form bone and thus a solid osseous construct, rather than fibrous tissue. Whilst it is straightforward to encourage differentiation into the osteogenic lineage *in vitro* by modulation of growth conditions and media constituents, many of these methods (e.g. the addition of dexamethasone, or the creation of a hypocapnic environment) are not transferable to clinical practice due to local and systemic side effects. However there is potential to use non-toxic factors *in vivo* to enhance cellular adhesion and encourage proliferation down an osteogenic lineage, resulting in improved mechanical and biological properties of the IBG composite.(Baas et al 2008).

In vitro work has shown that SSCs combined with allograft can not only survive the impaction process and continue to proliferate, but also improve the mechanical (shear) characteristics of the construct (Bolland et al. 2006). *In vivo* (murine) studies have reinforced these findings and also show enhanced new vessel formation within the constructs (Kanczler et al. 2010), raising the possibility of *de novo* bone formation and potentiation within the graft tissue. The accepted method for replacement of allograft in IBG is via creeping substitution; however the concept of a living graft which becomes incorporated into, rather than replaced by the surrounding host tissue presents a paradigm shift to the concept of allograft use. This would bring the advantages of autograft to the application of allograft, whilst avoiding any inherent donor site morbidity.

Chapter 2

Materials & Methods

2:1 Allograft

Fresh femoral heads, which are normally discarded after total hip replacement surgery were retrieved from patients after appropriate consent and local ethics committee approval. These were then stored at -80°C for over 6 months before processing. Under aseptic conditions, in the operating theatre the femoral heads were defrosted with warm saline. The soft tissue, articular cartilage and any osteophytes were removed from the femoral heads. Thick cortical bone was also removed using nibblers and an oscillating saw. The heads were then divided in two and placed into the bone mill. Previous studies have looked at a number of surgical bone mills (Dunlop, Brewster et al. 2003) and the Aesculap 3mm bone mill was found to give the optimum bone graft chip size with a well graded mixture. The milled allograft was placed in a jug and submersed in a solution of 6% Hydrogen peroxide for 10 minutes which acted as a de-fatting process. The layer of fat was decanted from the top of the jug and the remaining H_2O_2 poured out. The allograft was then subjected to repeat washing with pulsed lavage saline until the solution ran clear. Any further soft tissue or cortical bone noted at this stage was also removed.

Fig 2:1 Aesculap Bone mill with morcellised graft prior to washing and de fatting. Scale 5cm. Photo courtesy of Aarvold A.

The allograft was then soaked in an antibiotic / antimycotic solution for 24Hrs before further washing in a phosphate buffer solution. The graft was then dried prior to use.

2:2 Tissue culture

Cell Culture / Histology / Biochemistry

All cell based laborotary work was carried out with the assistance of the Bone and Joint Research group, with well established protocols for the majority of the steps outlined in this project. The IBG impaction and cell culture and mechanical testing were performed using techniques and materials as previously described by Mr S Tilley, Mr B Bolland and Dr A New. Summary of the main methods is as follows:

2:2:1 Human bone marrow stromal cell culture

As with the collection of femoral heads, bone marrow samples were collected from haematologically normal patients undergoing total hip replacements after appropriate consent and local ethics committee approval. In a 50ml falcon tube the bone marrow samples were repeatedly washed with -MEM and agitated to displace any cells from within the small bone marrow chips. The solution was then passed through a 70µm filter to collect any further bone or soft tissue debris. The falcon tubes were then centrifuged at 1100rpm for 5 min at $4^{o}C$. The supernant was discarded and the cell pellet resuspended in 10mls -MEM. The sample cell count was determined via a standard haemocytometer with the red blood cells lysed with a 1:1 solution of 0.1% acetic acid. The samples were then transferred to tissue culture flasks (number and size dependant on the sample yield) and cultured in -MEM and 10% FCS under either basal or osteogenic conditions (100uM ascorbate-2-phosphate, and 10nM dexamethasone) depending on the experimental run, and incubated at $37^{o}C$ in 5% CO_2. The first wash in PBS and media change occurred at day 6 and then was repeated every 3-4 days.

2:2:2 Cell passage

Upon reaching confluence, the cells were incubated for 10mins with a 10% Trypsin-EDTA solution in PBS. This caused the cells to release from the tissue culture plastic flask. For those cells cultured under osteogenic conditions, a further step of addition of Collagenase was added first in order to break down the extracellular matrix prior to Trypsinisation and prevent clumping of the cells. -MEM was added to the lysate to terminate the effect of Trypsin. The cell suspension was centrifuged at 1200rpm for 5 minutes at 4^oC producing a cell pellet. The solution of Trypsin and -MEM was removed from the pellet and the cells were then resuspended in fresh -MEM media (containing 10% serum) and either plated to culture flasks or seeded onto scaffolds depending on the experimental run.

2:2:3 Cell seeding & agitation

Cell count was performed using a standard haemocytometer. The volume of suspension required for the desired number of cells was calculated. The set quantity of allograft was contained in loose chip form within a falcon tube and the cell suspension was then added over the allograft in a drip wise fashion ensuring equal spread over the graft. Further plain media was then added to ensure complete graft coverage. The seeded allograft contained within the Falcon was then placed on an agitator (Belly dancer) within the incubator for 1 hour to allow the cells to mix freely with the allograft and adhere to the scaffold prior to impaction. Only P1 cultured cells were used in all studies.

2:2:4 Cell viability

Cell viability following impaction and culture was determined by using the fluorescent probes Cell tracker Green[TM] CMFDA (10µg/ml) for live cells and Ethidium Homodimer-1 (5µg/ml), (CTG/EH-1) for dead / necrotic cells. The cells were initially bathed in -MEM containing CTG/EH-1 at 37^0C for 1 hour. Samples were then washed twice with -MEM, bathed for a further 45 minutes (in -MEM only) and repeat rinsed in PBS before fixing in 4% paraformaldehyde for 12 hours. Samples were immersed in PBS and visualised with appropriate fluorescent filters.

Images were captured and processed with Carl Zeiss Axiovision software Ver 3.0 via an AxioCam HR digital camera on an Axiovert 200 inverted microscope (Carl Zeiss AG, Germany).
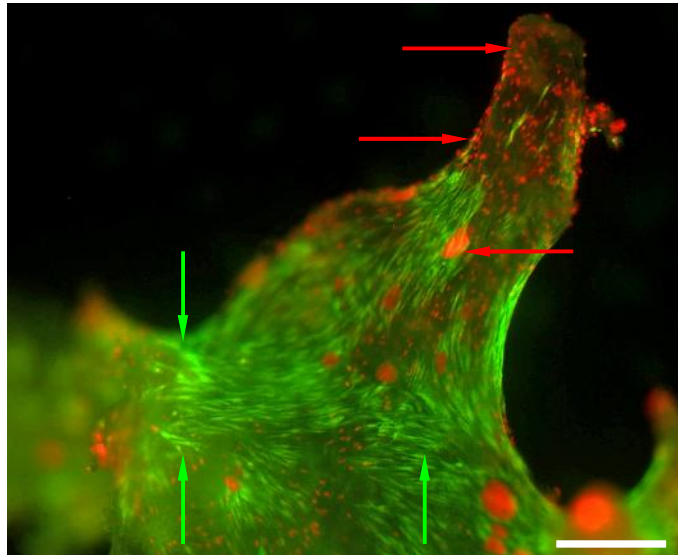
Fig 2:2 Fluorescent microscopy image of human allograft sample (baseline green colour) covered in CTG stained live SSC (vertical green arrows) and necrotic dead cells fluorescing red (horizontal red arrows). Scale Bar 100µm

2:3 Biochemical analysis

2:3:1 Alkaline phosphatase activity

Alkaline Phosphatase (ALP) enzme activity can be used as a marker for Osteogenic differentiation of SSC as it is expressed by osteoblasts during the early phase of bone formation. For this study the following assay was used to quantify the level of ALP activity.

Trypsin / EDTA (0.05%) was used to cover the samples and incubated at 37°C and 5% CO_2 for approximately 30 minutes with the samples vigorously vortexed twice during incubation. The solution was removed and cells were collected by centrifugation at 13,000rpm for 10 minutes at 4°C, and then resuspended in 1ml 0.05% Triton X-100. Cell wall Lysis was achieved by multiple freeze–thawing (>x3) and samples were stored at -20°C until assayed. 10ul of cell lysate from each sample was used to quantify alkaline phosphatase activity. Cell solutions were incubated at room temperature with 90ul of 2-amino-2-methyl-1-propanol buffer containing 100mM p-

nitrophenolphosphate (pNPP). The reaction was timed and stopped with 100ul of 1M sodium hydroxide (NaOH). Each sample was measured in triplicate against two standards. The turnover of pNPP, used to quantify alkaline phosphatase activity, was measured by the absorbance values at 410nm on a Bio-Tek KC4 Kineticalc microplate fluorescent reader. Results were expressed as nM pNPP/ng DNA/hr.

2:3:2 Pico Green DNA quantification assay

Measurement of DNA concentration was assayed with PicoGreen® dsDNA quantification reagent which is an ultrasensitive fluorescent nucleic acid stain that allows accurate detection of even small quantities of DNA. 10ul of cell lysate samples as described in 2:3:1 were loaded with 90ul Tris-EDTA (Tris-HCL Ethylenediamine Tetra-acetic acid) buffer and 100µl of 1:200 dilution of PicoGreen® in Tris-EDTA buffer. Samples were measured in triplicate against two standards. Flourescence was measured on a Bio-Tek Instruments, Inc. (USA) FLX-800 Microplate Flourescence reader using 480nm excitation and 520nm emission. Results were expressed as ng DNA/hr.

2:4 Histological analysis

2:4:1 Fixation and embedding

All samples were fixed with 4% paraformaldehyde, except for those undergoing alkaline phosphatase staining which were fixed in 95% ethanol.

In order to allow accurate sectioning all allograft samples were decalcified in EDTA / Tris solution prior to embedding. Conformation of decalcification was achieved by Imaging using a Faxitron® Specimen Radiography System MX-20 digital with Faxitron® Specimen DR software (Faxitron® X-ray Corporation, USA). Samples were then suspended in PBS solution prior to embedding. For paraffin embedding, samples were first dehydrated in graded ethanol solutions then placed in 1:1 ethanol/chloroform solution, and finally in chloroform twice for 1 hour before being soaked in paraffin wax at 60°C twice. Samples were embedded in hot wax and placed on a cooling surface to solidify.

2:4:2 Slide preparation

A Microm 330 microtome (Microm International GmbH, Germany) was used for sectioning. The Paraffin wax embedded samples were removed from cold room storage and trimmed of excess wax. 6µm sections were cut and placed on a water bath at 37°C and allowed to spread. Sections were captured onto pre-heated glass slides and then transferred to a drying oven at 37°C for 3 hours. Slides were stored at 4°C and were warmed to room temperature prior to staining.

2:4:3 Alcian Blue & Sirius Red staining

Histoclear was used to clear excess wax, the samples were then rehydrated by passing through graded methanol solutions. Residual methanol was removed in the water bath, and followed by nuclear staining with Weigert's haemotoxylin for 10minutes. Slides were rinsed in water and stained with Alcian blue for 10 minutes to demonstrate proteoglycan rich cartilage matrix. The slides were then treated with molybdophosphoric acid for 20 minutes followed by staining with 0.1% Sirius red F3B for 1 hour to demonstrate collagen. The sections were then dehydrated through reverse graded methanol and histoclear steps.

2:4:4 Alkaline phosphatase staining

Alkaline phosphatase enzyme activity is used as an early marker for osteogenic differentiation; samples were incubated with a colourimetric solution reactive to intrinsic alkaline phosphatase. Ethanol fixation of the samples was carried out prior to histochemical staining. Naphthol AS-MX phosphate and Fast Violet B salts were combined as per a Sigma-Aldrich protocol and applied to the culture samples. Following the development of a red-purple stain the reaction was stopped with distilled water. The samples were placed in cold storage until imaged.
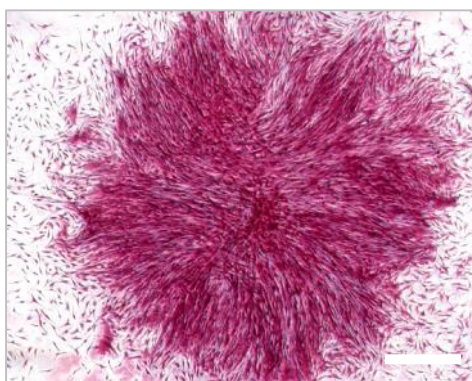
Fig 2:3 Microscope image of a single CFU-F on tissue culture plastic following 14 Days culture in basal media. Alkaline phophatase stain performed to highlight osteogenic differentiation (pink) of the cells cultured from a single progenitor cell. Scale 100μm

2:4:5 Immunohistochemistry

Initially any endogenous peroxidase activity was arrested with 3% H_2O_2 for 5 minutes; sections were then rinsed with water and blocked with 1% BSA in PBS for a further 5 minutes. Sections were incubated overnight at 4°C with the primary antibody. To remove any residual antibody, sections were rinsed with water and passed through wash buffers of high salt, low salt and tris for 5 minutes each. The appropriate biotin-conjugated secondary antibody was applied for 1 hour followed by further rinses in wash buffers. The secondary antibody complex was linked with Streptavidin peroxidise and incubated for 30 minutes. Samples were developed using 3-amino-9-ethyl-carbazole (AEC) in acetate buffer containing H_2O_2, to yield a reddish-brown reaction product. Nuclear staining with Weigert's haemotoxylin, and an appropriate counterstain was then applied followed by mounting in glycerol jelly. Negative control slides were performed by omitting the appropriate primary antibody.

2:5 Mechanical testing and IBG

2:5:1 Impactor design

Previous studies by Brewser et al, using force plate analysis have determined the total force imparted during a standard femoral impaction bone grafting to be equivalent to a 1.98kg mass falling 65mm onto a circular base plate 60mm in diameter (Brewster, Gillespie et al. 1999). The bioengineering department at Southampton University have used these values to rescale and produce custom impactors for both in vitro (large impactor) and in vivo experiments (small impactor). Both impactor designs were based on a free sliding drop weight dropping a set distance onto an enclosed chamber which could be sequentially filled with graft.
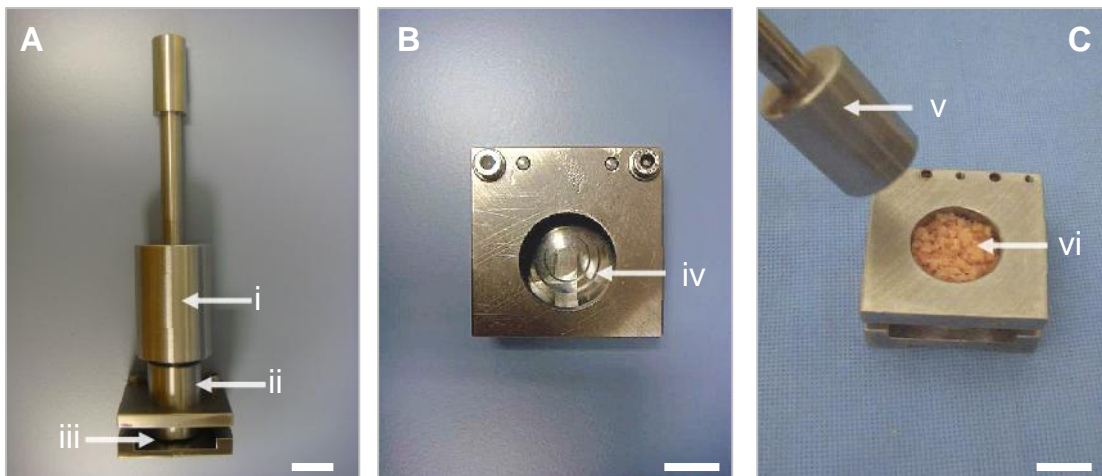
Fig 2:4 Photographs of Large impactor used for in vitro studies. A: (i) Drop weight on shaft. (ii) Impactor head engaged in base plate. (iii) Base plate with gap to accommodate acrylic ring for graft containment. B: (iv) Base plate with central opening through which the graft is introduced into. C: Base plate and Impactor after final impaction (v) Impactor head. (vi) Base plate with central hole now filled with impacted allograft. The acrylic ring containing the allograft is then carefully slid out of the base plate and an acrylic base and lid added. Scale bars 20mm approx. The in vivo impactor was a similar design but scaled down.

2:5:2 Allograft Impaction

Using the modified impactors described in 2:5:1, Impaction of allograft was carried out as described by Dunlop et al (Dunlop et al., 2003). The milled and seeded allograft was introduced into the top of the impactor chamber in three equal portions, to ensure even compaction. The weight was dropped 24 times from the set height at a rate of approximately 1Hz for each portion of allograft so that the finished pellet received a total of 72 impactions.

2:5:3 Shear testing

A custom made cam shear tester was used for all shear testing of the samples as per Fig 2:5. The graft was carefully transferred into the chamber and an axial load applied for 5 min before the shearing process began. Axial loads of 50N, 150N or 250N were used giving compressive stresses of 102kPa, 306kPa and 509kPa respectively which are within the physiological stresses encountered within the hip joint.
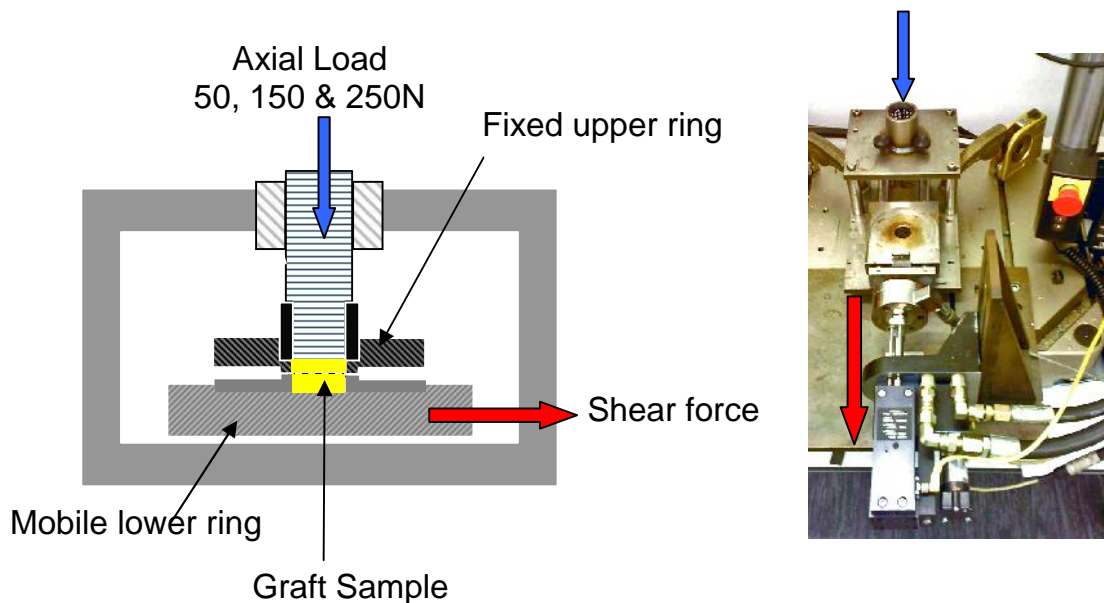
Fig 2:5 Schematic representation of the cam shear testing jig with photo of the set up on the right.. The disc of allograft is the central yellow square. There is a fixed upper ring and a mobile base plate that a constant shear force is applied via an actuator connected to the lower ring in the direction of the red arrow. Prior to shearing the sample an axial force is applied for 5 minutes at 3 different compressive loads (One per sample) of 50N, 150N or 250N as represented by the blue arrow.

Shear testing of the allograft under compressive loads gives rise to a family of stress strain curves as shown in Fig 2:6 with each curve representing a single sample. The graphs in Fig 2:6, 2:7 and 2:8 show the data from the cell concentration study as detailed in Chapter 3, to demonstrate how the linear regression plots are constructed from the base data.
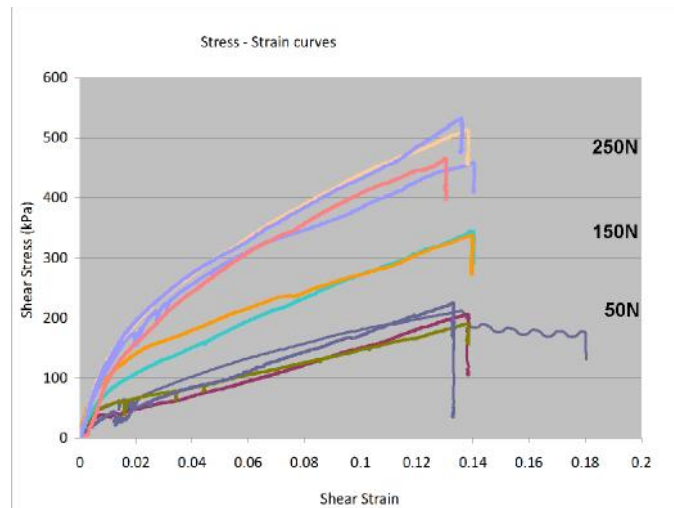
Fig 2:6 Graph showing an example of a Family of Stress Strain Curves produced from the mechanical shear testing. Each line representing a single samples behaviour. The three distinct groupings of the lines are due to the three different axial compressive loads 50, 150 and 250N

The shear strength value at 10% strain (an industry standard point) is then determined and plotted against the value of the axial load (Normal Stress kPa). See Figures 2:7 & 2:8.
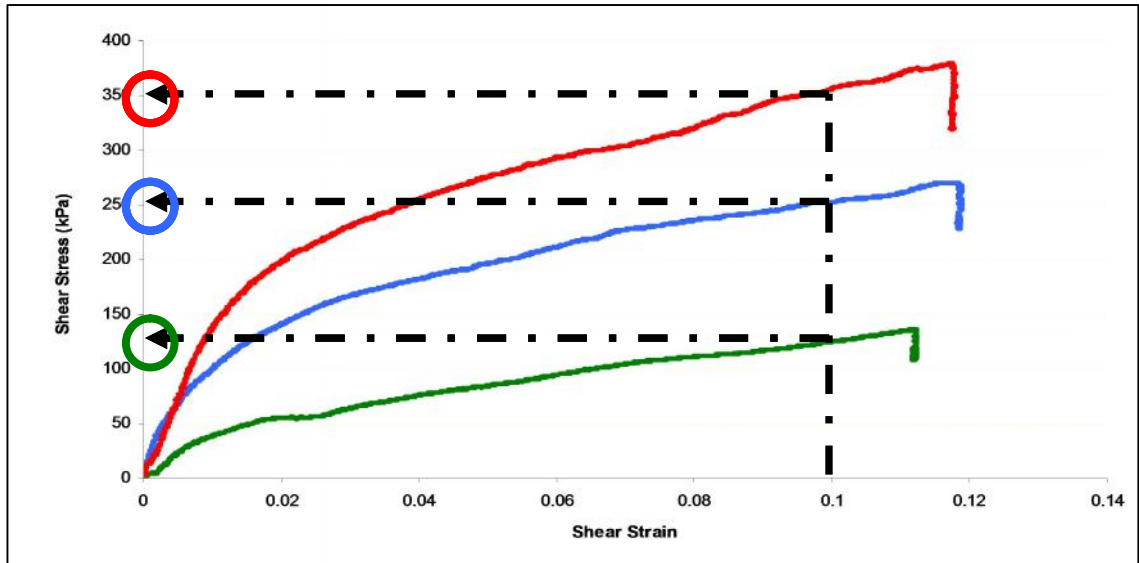
Fig 2:7 Graph showing 3 stress strain curves at increasing axial loads / normal stress. The shear stress at 10% strain is calculated by extrapolating back to the y values as demonstrated by the arrows. These values are then plotted against the axial load expressed as normal stress in kPa

Linear regression analysis can then be performed on the values to find a best fit line (Fig 2:8)
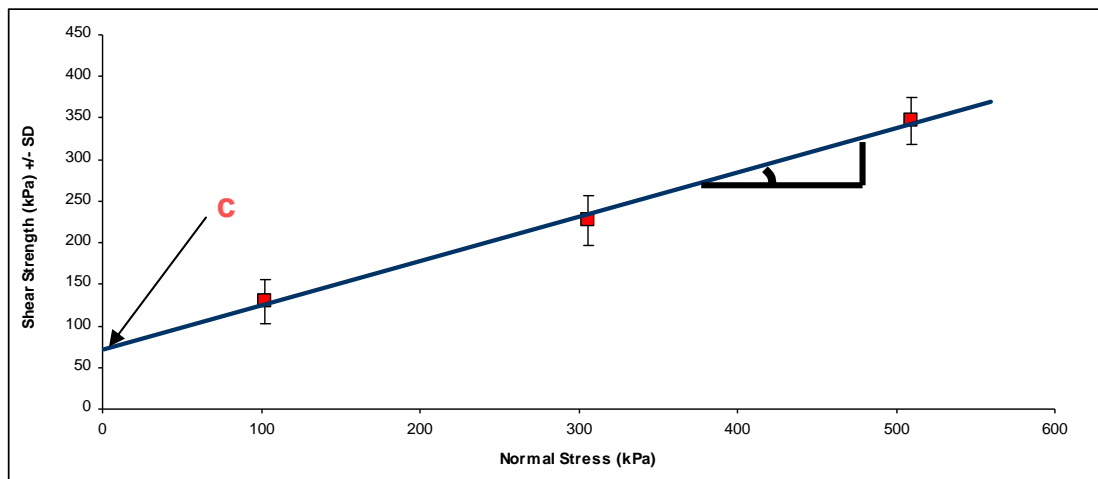
Fig 2:8 Graph showing the 3 points plotted on the graph. Linear regression analysis is then used to find a best fit line (blue on graph). c = y intersect = interparticulate cohesion value.   = angle of slope = internal friction angle

If the samples obey the Mohr Coulumb law i.e there is a linear relationship between the points (signified by a R^2 value close to 1) then the shear strength (y intercept for a given Normal Stress level), Interparticulate cohesion (c = y

intercept at 0kPa) and internal friction angle (angle of slope) can be calculated. **The Mohr Coulomb Law** is expressed as follows:

$$\textbf{Shear Strength} \quad = \quad \tan \quad + \textbf{c}$$

c = particle cohesion

  = axial stress

tan   = Internal friction angle

2:6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Ver 5). For comparisons of the means, a two sample or 'Student's' t-test was performed. Linear regression analysis was performed on all Shear strength data with correlation r^2 >0.98 for all. Comparison of multiple linear regression analysis was performed using Analysis of Co-variance (ANCOVA). A p-value of < 0.05 was taken as significant and divided in this thesis as either <0.05, <0.01 or <0.001. The number for all experimental runs is documented in the individual experiments, but n > 3 for all. Error bars are equal to +/- 1 Standard deviation.

SECTION I

The Biomechanical Effects of Skeletal Stem Cell Concentration in Impaction Bone Grafting

Chapter 3:

The Effect of Skeletal Stem Cell Concentration on the Biomechanical properties of Impacted Bone Graft

Chapter 4:

Clinical Translation of Enriched Skeletal Stem Cell Cells Seeded Onto Allograft in the Treatment of Fracture Non Union and Avasular Necrosis of the Hip

Chapter 3

The Effect of Skeletal Stem Cell Concentration on the Biomechanical properties of Impacted Bone Graft

3.1 Introduction

Following increasing patient numbers undergoing primary hip arthroplasty, an increase in patients requiring revision arthroplasty surgery has been seen. In the UK between 2003 and 2008, the number of revision hip procedures performed doubled to almost seven thousand per year (NJR 2008). Many of these patients may have received their original implants when joint replacement surgery was in its infancy and the techniques, prostheses and instruments had not yet been refined. Consequently, these patients now present in increasing numbers with extensive bone loss around loose implants posing a significant orthopaedic challenge for revision surgery.

With almost a forty year history in revision hip surgery, impaction bone grafting (IBG) using fresh frozen morcellised allograft remains the current gold standard for replacing lost bone stock (Schreurs et al. 1998). This technique endeavours to recreate a solid, adaptive mantle into which the implant can sit securely, immediately able to withstand forces associated with normal implant use. The ability of the graft bed to resist shear and go on to form a new living bony construct is a critical factor in the long-term success of the revision implant. The results are encouraging, with up to 87% twenty year survivorship for acetabular components and ten year femoral component survival of 99%(Schreurs et al. 1998; Halliday et al. 2003) However, widespread adoption of this demanding technique has not always led to a repeat of these results outside of the major centres (Halliday et al. 2003), and thus laboratory based research endeavours to find techniques to improve the desirable characteristics of the allograft.

Previous in vitro studies have identified both mechanical and biological factors that can be modulated to augment the structure and performance of IBG. Factors, such as preparation of graft, constituent particle size, chemical additions and local fluid drainage have all been shown to affect the performance of IBG (Dunlop et al. 2003). The combination of skeletal stem cells (SSC) with the allograft has become an area of particular interest for the enhancement of impacted allograft. In vitro work has shown that SSC's combined with allograft can not only survive the impaction process and continue to proliferate, but also improve the mechanical (shear) characteristics (Bolland et al. 2006). In vivo (murine) studies have reinforced these findings, but also show enhanced new vessel formation within the constructs and hence raise the possibility of de-novo bone formation and survival within the graft tissue (Kanczler and Oreffo 2008).

The accepted method for replacement of allograft in IBG is via creeping substitution. The concept of a living graft which becomes incorporated into, rather than replaced by the surrounding host tissue presents a paradigm shift to the concept of allograft use.

The beneficial clinical effect of autologous bone marrow has been clearly demonstrated by Hernigou et al in the treatment of fracture non-unions and femoral head osteonecrosis (Hernigou, Poignard et al. 2005). For atrophic non union cases it was established that the concentration of the aspirated marrow was necessary to produce a therapeutic effect was above 1000 CFU-f/ml and that the mean concentration in bone marrow aspirate is approximately 600CFU-F/ml. Whilst the use of SSC in clinical practice has been established for non unions, treatment of AVN and in the addition to IBG for acetabular reconstruction (Deakin and Bannister 2007) the clinical results have been variable. This may be attributable to the variable concentration of SSC within the bone marrow aspirate as noted by Hernigou. To my knowledge, no work has been performed to establish the optimal SSC concentration for use in impaction bone grafting.

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3:2 Null Hypothesis

The seeding density of SSC on to allograft has no effect on the biomechanical properties of impacted bone graft

3:3 Aims

This aim of this study was to examine both the biological and mechanical characteristics of impacted allograft when combined with increasing concentrations of SSC's and to analyse the effects of increasing SSC seeding density on the IBG Shear strength

3:4 Study design

3:4:1 Cell Culture & Graft Preparation

As previously described in 2:1 and 2:2. P1 Culture expanded SSC were used for all experimental runs harvested from the waste marrow of haematologically normal patients undergoing elective THR. Cells were split off the culture flasks as described and the concentration established via haemocytometer.

3:4:2 Graft Seeding

The allograft was seeded at 3 different SSC concentrations (5×10^3, 5×10^4 and 2×10^5 cells / cc allograft) along with plain allograft as a negative control, and the experiment was repeated for each cell concentration (and plain allograft control), giving two data sets for each cell concentration. These seeding densities were used as previous experiments by Bolland et al have demonstrated that 2×10^5 cells per cc allograft was sufficient to cause a significant increase in the constructs shear strength. This has therefore established an upper limit, and this concentration was repeated in my study to confirm reproducibility of the experimental set up. The lower concentrations of 5×10^3, 5×10^4 were used to refine the experiment further to try and establish the lowest concentration required to cause a significant improvement in the shear strength of the IBD / SSC construct.

Experimental Groups:

- Control - Plain Allograft
- 5000 SSC / cc Allograft
- 50000 SSC / cc Allograft
- 200000 SSC / cc Allograft

Allograft was prepared in standard fashion as previously described and aliquoted into 10cc and placed in a falcon tube. The desired amount of SSC were resuspended in -MEM and then added to the allograft in a drip wise fashion as described in section 2:2:3. The time was limited to 1 hour in order to simulate time that would be available intra-operatively, thereby making this technique highly relevant for clinical translation.

3:4:3 Graft Impaction

Using the larger impactor as described in section 2:5:1, all samples underwent 3 stage impaction with a total of 72 blows at 1Hz. The compacted discs were then contained in custom made acrylic discs to keep the allograft contained, with multiple pores across the whole surface to allow free flow of culture media in and out of the samples.

3:4:4 Tissue culture

The contained acrylic discs were then submerged in culture media within sterile pots and placed in an incubator. Regular media changes were performed and the discs cultured for a total of 2 weeks as this had been previously shown to be an adequate culture period to show any potential differences (Bolland et al. 2006). A longer culture period significantly increased the chance of infection with further cost and time implications and was not felt to be required for this primary study. Once culture was complete, the samples were sealed within their containers, in culture media and transferred to the mechanical testing jig within 3 hours.

3:4:5 Mechanical shear testing

A custom made cam shear testing rig was set up as described by Bolland et al. and fitted into an Instron testing machine (Instron, High Wickham, UK). This composed of a chamber between two shear plates within which the graft was placed (See section 2:5:3). Eighteen of the impacted discs were subjected to a constant shear strain of 1mm/min whilst under 3 different compressive stresses of 50N, 150N and 250N (6 discs per stress), and from this data shear strength, interparticulate cohesion and internal friction angle parameters were calculated using the **Mohr Coulomb** failure equation:

$$\textbf{Shear Strength} \quad = \quad \tan \quad + \textbf{c}$$

3:4:6 Cell viability

Cell viability of the samples was assessed at day 0 to confirm attachment of the cells and survival post IBG. Further analysis of cell viability was carried out following 2 Weeks of incubation. This was achieved using Cell Tracker Green probe and Ethidium Homodimer-1 as described in section 2:2:4.

3:5 Results

3:5:1 Mechanical shear testing

Linear regression analysis of the shear data gave an R^2 value >0.99 showing that a close linear relationship existed and confirming that the samples were suitable for further analysis. Using the Mohr Coulumb equation we can then calculate the interparticulate cohesion of the samples.
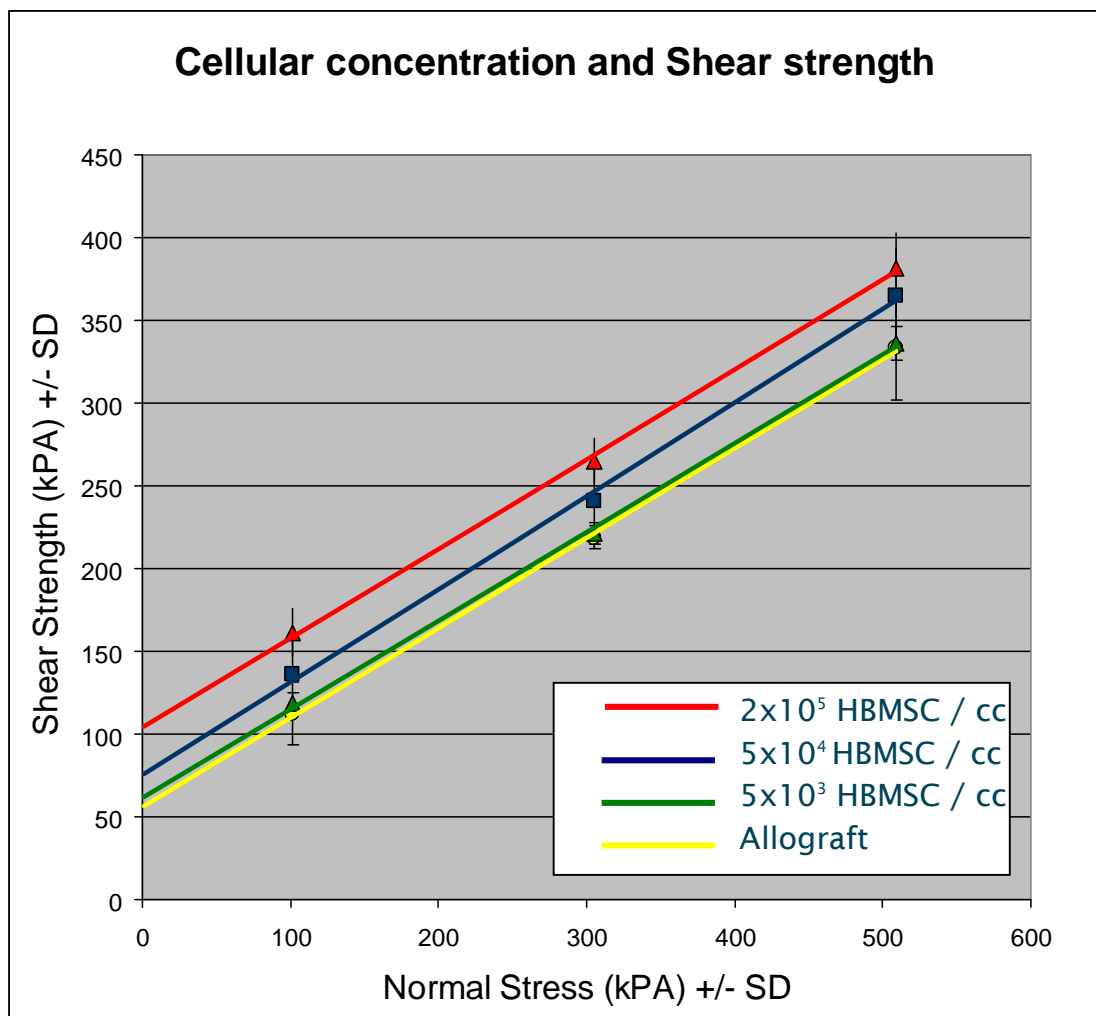
Fig 3:1. Linear regression analysis of the shear stresses at 10% Strain demonstrating progressive increase in shear strength and interparticulate cohesion with increasing SSC concentration

As shown in Table (3:1) and Graph (3:1) (c = y axis intersection) this progressively increases with increasing cell seeding density. The shear strength of the construct can also be calculated, in table (3:1) the values at 350kPA are shown with 350kPA being within the normal physiological load of a hip joint. This was approaching significance at $5x10^4$ cells/cc (p=0.06), and highly significant at the $2x10^5$ level (p=0.001).

Cell Concentration (cells/cc allograft)	R^2 value for linear regression	Shear Strength At 350kPa	Interparticulate Cohesion c kPa	Significance Grouped linear regression analysis
Plain Allograft	>0.99	245.5	55.8	vs Plain Allograft
$5x10^3$	>0.99	248.9	61.3	p=0.76
$5x10^4$	>0.99	272.2	75.6	p=0.06
$2x10^5$	>0.99	284.9	95.7	p=0.001

Table 3:1: The effect of increasing SSC concentration (cells/cc allograft) on Shear strength ( ) and Interparticulate cohesion(c)

3:5:2 Cell viability

Figure 3:2 shows good viability of cells on the seeded allograft at day 0 demonstrating adherence of the cells to the scaffold, and survival of the impaction process. It also confirms no contamination of any live cells in the control group. After 2 weeks incubation the live cell density (shown by the bright green areas) clearly increases in keeping with ongoing cell division and differentiation. In the two higher seeding densities new matrix formation can be seen starting to bridge the gaps between the allograft chips.
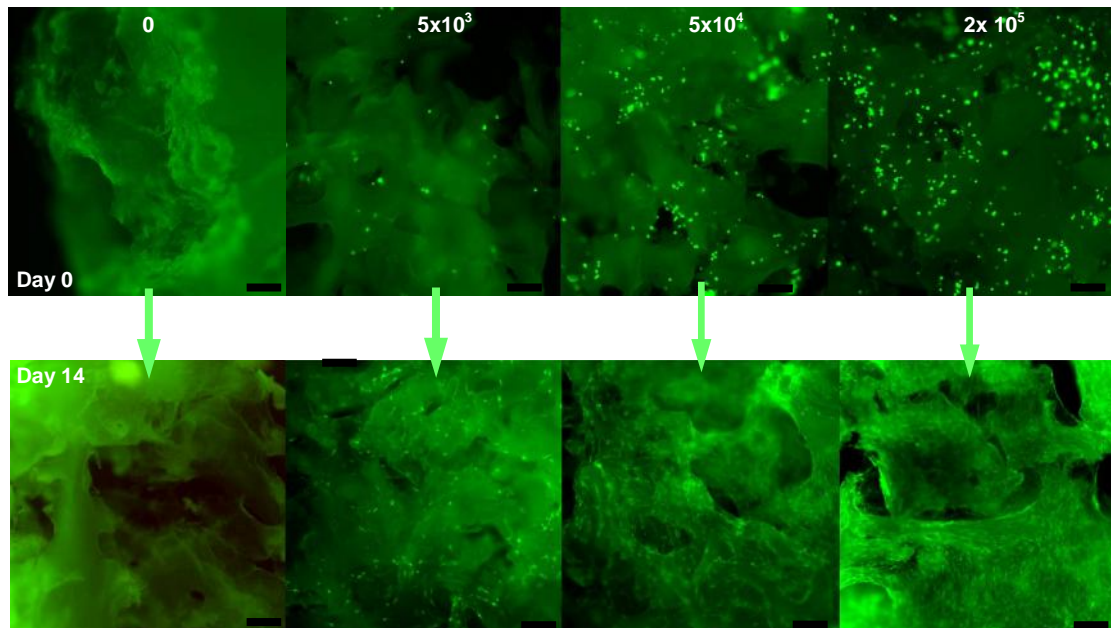
Fig 3:2 CTG live staining of samples at Day 0 post impaction and following 14 days of culture. Scale bar 200μm

3.6 Discussion

This study shows that skeletal stem cell number has a measurable effect on the impacted allografts' mechanical properties at two weeks incubation. The shear strength of the impacted allograft increases in combination with increasing concentration of seeded SSC. At $5x\ 10^3$ there is no significant difference, but this is approaching significance at a concentration of over $5x10^4$ cells/cc of allograft. There is a 16% increase in strength of the disc as a whole when seeded at $2x10^5$cells/cc, and this turns out to be a 72% increase in the interparticulate cohesion. If we correlate the mechanical results to the Live dead staining of the samples we can see that for the lowest seeding density $(5x10^3$/cc) at 2/52 the cell and matrix coverage of the allograft is still fairly sparse. In the higher concentration groups there has been more cell proliferation and subsequent matrix formation and it is surmised that this bridging between the bone graft chips of the cells and ECM increase the cohesion between them, thus improving their shear strength.

Previous work performed in our department reinforces the mechanical advantage of SSC seeded allograft compared to allograft alone, but there has been no published research to date which investigates the effect of increasing

cell seeding concentration in the IBG example. However, when investigating the treatment of established tibial non-unions, Hernigou et al. injected the site with concentrated aspirated bone marrow (Hernigou et al. 2005; Mont, Zywiel et al. 2010). An increase in efficacy of the aspirate in promoting union was found when the SSC concentration was increased by centrifugation of the sample, demonstrated by parallel in vitro CFU-F counts. Of 60 patients treated by this method, 53 progressed to union, and the seven persitent non-unions all had lower CFU-F counts.

SSC's are rare in bone marrow, representing less than 1 in 10,000 nucleated cells. Given our finding that mechanical advantage in the IBG model is only gained at concentrations of over 5×10^4 cells/cc allograft, this also has important implications when translating this work to theatre as it is clearly important to know at which concentration the cells need to be seeded, in order to confer the advantage to the patient as soon as possible. Clinically patients can be weight bearing on the affected limb from very early in the post-operative period, hence a clear increase in the construct strength from two weeks will reduce the risk of stem subsidence as well as failure (in shear) from an early stage. However, with this mechanical advantage requiring in the order of 10^7 cells at operation (2 femoral heads = approximately 200mls allograft), there are not enough SSC's present in a pelvic aspirate to provide this number of cells. Hence to translate this clinically there are three options:

i)   In-vitro culture of autologous SSC's, such that the numbers required for seeding can be produced. This will require an additional procedure several weeks before the revision hip surgery.

ii)   Intra-operative concentration (eg by centrifugation) of autologous bone marrow aspirate.

iii)   The use of donor SSC's. This would allow the production of 'off the shelf' seeded allograft, but risks infective transmission as well as rejection.

The benefits of SSC seeded allograft are however not only mechanical. Previous work by our group with biochemical and histological analysis has shown continued cell survival and proliferation after two weeks in culture. Col 1 staining and ALP specific activity also indicates osteoblastic differentiation. Previous studies by our group have also shown the induction of neovascularistion by SSC seeded allograft in a murine model (Bolland et al. 2007). It is important that this occurs early during graft incorporation, to ensure oxygen and nutrients can be provided to the proliferating cells hence when translated to the clinical environment it can be hypothesised that the graft itself will survive and replace the lost bone stock, rather than being replaced by the surrounding bone, which can take in excess of 18 months. Furthermore, this technology can be translated to other areas of orthopaedics, including fracture non-unions, segmental bony defects and avascular necrosis.

3:7 Summary

The combination of SSC's with allograft provides interesting and clinically translatable results. They appear to enhance the mechanical characteristics of the allograft, as well as providing the opportunity for graft survival and incorporation. I have now shown that increasing cell seeding density further enhances this activity and should be more than 5×10^4 cells/cc allograft.

3:8 Conclusion

The results in the context of the null hypothesis were examined:

Null Hypothesis
The seeding density of SSC on to allograft has no effect on the biomechanical properties of impacted bone graft

This hypothesis is false

Chapter 4

Clinical Translation of Enriched Skeletal Stem Cell cells seeded onto allograft in the treatment of non union and AVN of the hip.

4:1 Introduction

In chapter 3 I have demonstrated that SSC seeding density plays a critical role in the in vitro biomechanical strength of the IBG construct, but that this only occurs at a relatively high seeding density, and certainly significantly higher than that found in bone marrow aspirate In order to transfer the maximal biomechanical advantage of the addition of SCC to the IBG construct to prevent subsidence or failure it is therefore necessary to use a highly concentrated SSC sample. This chapter describes the translation of these in vitro findings to the treatment of a number of orthopaedic conditions with the use of a centrifuge based concentration system. Parallel in vitro analysis of the samples is also undertaken.

Fracture non union can be caused by a number of conditions, both patient factors such as diabetes, or smoking and mechanical factors such as inadequate stability or bone loss / fracture gap. The non unions can be broadly classified as hypertrophic or atrophic. Atrophic non union occurs when the fracture ends loose any potential for callus formation / osteogenesis usually due to a loss of local blood supply in the early phase of fracture healing (Reed A et al 2003). It has been demonstrated that Skeletal stem cells can induce angiogenesis (Kanczler and Oreffo 2008) and if used in combination with a scaffold may be able to induce union of the fracture. The SSC concentration has also been shown by Hernigou to be a critical factor (Hernigou et al. 2005).

Avascular Necrosis (AVN) of the femoral head is a disease that usually affects young patients with the average age in the USA requiring THR for complete collapse being 38 (Solacoff D et al 1993). Causes of AVN can be either post

traumatic or non-traumatic with over 90% of these cases due to Steroids or Alcohol excess (Mont et al 1995). Other causes include Sickle Cell Disease, chemotherapy, radiotherapy and coagulation disorders. It is a particularly debilitating condition in that untreated, over 80% of patients will progress on to osteoarthritis (Mont, Carbone et al. 1996) and even in the asymptomatic there is a 59% chance of progression (Mont, Zywiel et al. 2010).

The Ficat and Arlet is the most widely used system for staging the pathology of the disease (Ficat 1985).

Ficat and Arlet Staging:

Stage 0

- o X-ray : normal
- o MRI : normal
- o clinical symptoms : nil

Stage I

- o X-ray : normal or minor osteopaenia
- o MRI : oedema
- o Bone scan: increased uptake
- o clinical symptoms: pain typically in the groin

Stage II

- o X-ray : mixed osteopaena &/or sclerosis
- o MRI : geographic defect
- o Bone scan : increased uptake
- o clinical symptoms: pain and stiffness

Stage III

- o X-ray : crescent sign & eventual cortical collapse
- o MRI : same as Xray
- o clinical symptoms : pain and stiffness+/- radiation to knee and limp

Stage IV

- o X-ray : end stage with evidence of secondary degenerative change
- o MRI : same as Xray
- o clinical symptoms : pain and limp

There are a number of other scoring systems but the main crux of the diagnosis and staging are whether the femoral head sphericity is maintained (Stage 1 and 2) or if there has been collapse and loss of the bony architecture (Stage 3 & 4). Once there has been collapse of the head, the treatment options become fairly limited with rapid progression to OA and subsequent THR as appropriate. For the earlier stages of the disease, prior to collapse of the femoral head, there are a wide number of treatment modalities that have been used in an attempt to arrest the seemingly inevitable disease progression. These range from simple protected weight bearing for small areas of AVN with Cheng et al 2004 showing spontaneous resolution of some lesions. More prolonged follow up by Henigou et al 2004 and metaanalysis of 819 non operatively treated hips by Mont MA however have shown that 73 -75 % will eventually progress and require operative intervention, so protected weight bearing is no longer recommended. Phamacological treatment has also been studied, with the bisphosphonate: Alendronate used to treat 100 hips (Agarwala et al 2005) by preventing osteoclastic bone resorption. They demonstrated clinical improvement in outcome disability scores at 1 year mean follow up with only 10% requiring surgical intervention. It should be noted that the follow up length was a mean of 1year and 5 years maximum, so again as per Hernigou's study a significant proportion of these may go on to collapse.

Core decompression of the femoral head has been used as a surgical treatment for AVN for a number of years. The principle behind the technique is that it decompresses the head, reducing intramedullary pressure and removes the necrotic area of bone. A metaanalysis of 1206 hips that underwent core decompression showed an overall clinical success rate of 63.5% (Mont MA et al 1996) with less than 33% requiring replacement or salvage procedure at last follow up. Core decompression doesn't provide any initial mechanical support however, and relies on angiogenesis and new bone formation from the surrounding tissues, and it has been well documented that in AVN the osteoprogenitor numbers are reduced (Hernigou et al. 1999; Calder et al. 2001).

Porous Tantalum implants used in combination with core decompression has also been used with the Tantalum strut providing the mechanical support and allowing bony in growth into the implant. Prospective analysis of 60 hips treated with this method by Veillette et al (2006) showed a survival of the femoral head in 68% of cases at 4 years follow up. A smaller study of 22 hips by Shuler et al (2007) showed 86% survival at a minimum 2 year follow up of grade 1 and 2 disease. These results are encouraging, but have the disadvantage of using a metal implant rather than replacing the native bone stock which may lead to more demanding surgery should the need for revision to THR be required.

Vascularised free fibula grafting for hip AVN could be considered the gold standard treatment, as it is replacing the necrotic area of bone with native living bone and providing immediate structural support and an established new blood supply. The results of this technique from a number of studies involving over 300 hips in total (Zhang 2000; Marciniak 2005; and Yoo et al 2008) all show significant improvements in Harris hip scores at last follow up with only 10% requiring conversion to THR at 10 years follow up in Yoo et als study. Although the results from Marciniac et al are less favourable with 61% and 42% survival of the femoral head at 5 and 8 years respectively. It should also be noted that the surgical technique for this method is both technically demanding and time consuming and in the UK would be likely to require the input of a reconstructive / plastic surgeon as well.

More recent work by Hernigou (Hernigou et al 2009) and others have used SSC and or Allograft IBG, but to our knowledge no study has been done looking at the combination of concentrated SSC and IBG for the treatment of AVN.

4:2 Aims

The purpose of this study was to see if the findings from the previous chapter of increased biomechanical strength with increasing SSC concentration can be clinically translated to improve patient outcome for a number of conditions.

4:3 Null Hypothesis

(i) The use of a centrifuge concentrated bone marrow aspirate in combination with impacted allograft does not improve the treatment of fracture non union or AVN of the hip

(ii) Parallel in vitro culture of the samples does not demonstrate any biological advantage compared to the unconcentrated sample.

4:4 Clinical Methods

4:4:1 Patient Cohort

Fracture non-union:

one case was identified as a suitable candidate for the procedure. The patient was a 72 yr old female who had originally undergone a Right THR complicated by a sciatic nerve injury. She had significant co morbidities of diabetes, hypertension, hypercholesterolemia and had a high Body Mass Index. As a result of her sciatic nerve palsy she fell and sustained an intraarticular bicondylar distal femoral fracture. (Fig 4:1)
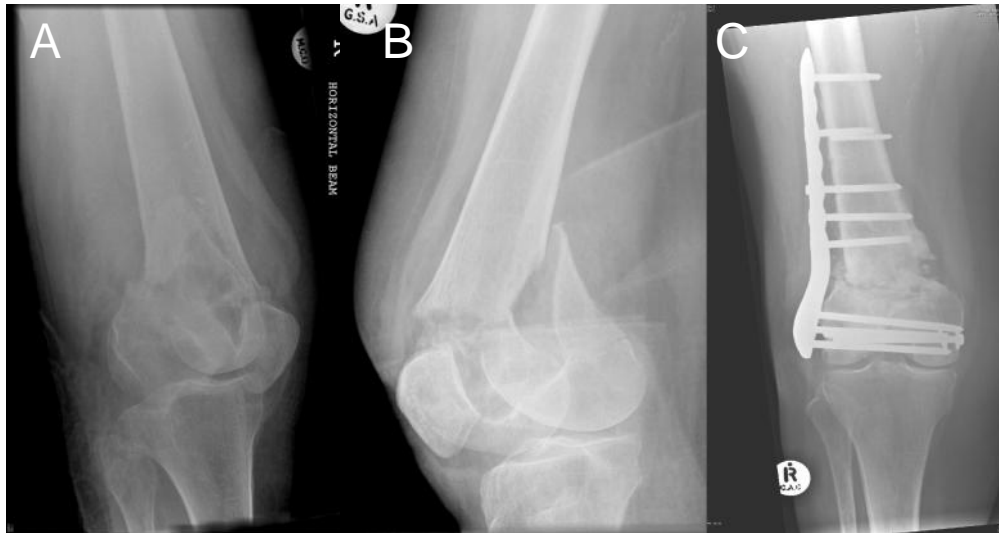
Fig (4:1) Plain radiographs demonstrating A (AP) and B (LAT) of Distal bicondylar T shaped fracture. C: Second plating with contoured locking plate after initial metalwork failure. Note fracture line still clearly visible

This was initially treated with a DCS plate which subsequently failed and was revised to a locking distal femoral plate (locked proximaly and distally). Despite adequate reduction and good fixation of the fracture, it failed to unite with no change in the xray apearence over 4 months and a CT scan Fig (4:2) confirmed non union. There was no clinical or haematological signs of infection.

Fig(4:2) Coronal CT scan of distal femur showing established atrophic non union.

4:4:2 Avascular Necrosis:

A total of four cases of femoral head AVN were treated as part of this study. The basic patient parameters were as follows

Patient	Age	Attributed Cause	Right hip stage*	Right hip treatment	Left hip stage*	Left hip treatment
1	42	Systemic steroids for sub-arachnoid haemorrhage	IV	THR	II	SSC/IBG
2	40	Alcohol	III	THR	II	SSC/IBG
3	31	Idiopathic	III	THR	II	SSC/IBG
4	32	Systemic steroids for testicular carcinoma	II	SSC/IBG	II	SSC/IBG

Table of patient parameters, aetiology and treatment

*Ficat & Arlet Stage (Ficat 1985)

4:4:3 Bone Marrow Harvest and Concentration

After informed consent under asceptic conditions in theatre a standard bone marrow aspiration was performed from the iliac crest. Due to the volume of bone marrow aspirate required (120ml), care was taken to try and gain maximum yield with minimal contamination from systemic blood. This was achieved by multiple passes of the Jamshee needle with only small volumes aspirated before twisting to 90Deg and withdrawing. In 3 of the 4 cases both iliac crests were utilised.
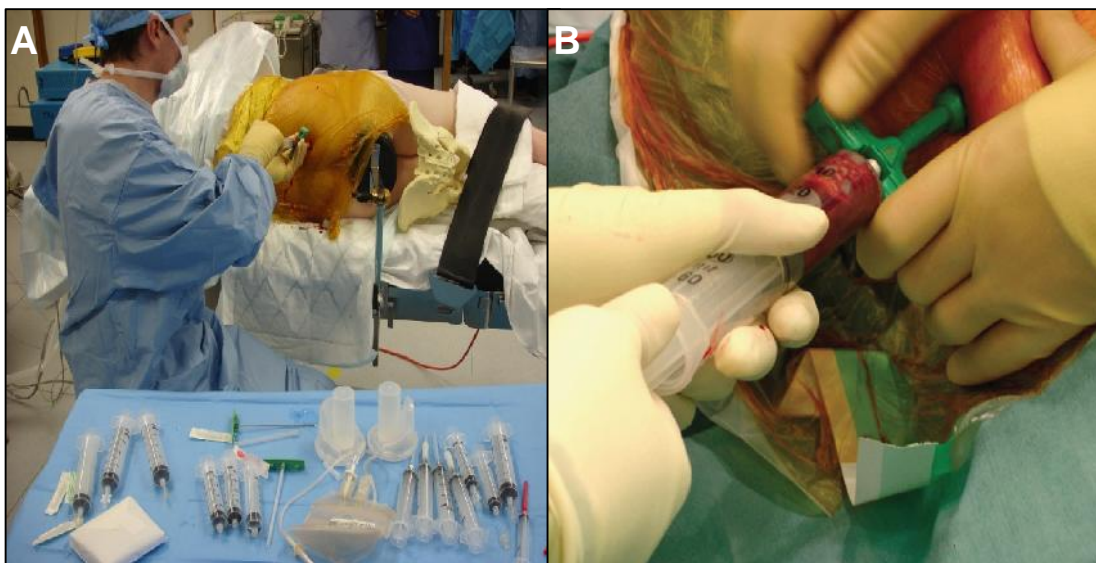
Fig 4:3 A: Aspirating the Rt iliac crest for bone marrow harvest. HARVEST syringes, filters and centrifuge pots can be seen in the foreground. B: Close up of the marrow aspiration via a jamshee needle cored into the iliac wing

Once 120ml of sample had been collected the bone marrow was anticoagulated with 8ml Anticoagulant Citrate Dextrose Solution A (ACD-A) as per the manufacturers guidelines. The sample was placed in a blood giving set and passed across a filter. The filtered sample was then carefully decanted into the centrifuge pots. This particular device worked on a floating shelf principle to separate the buffy coat from the red blood cell fraction. Other centrifuge systems were available and all worked on broadly similar principles.
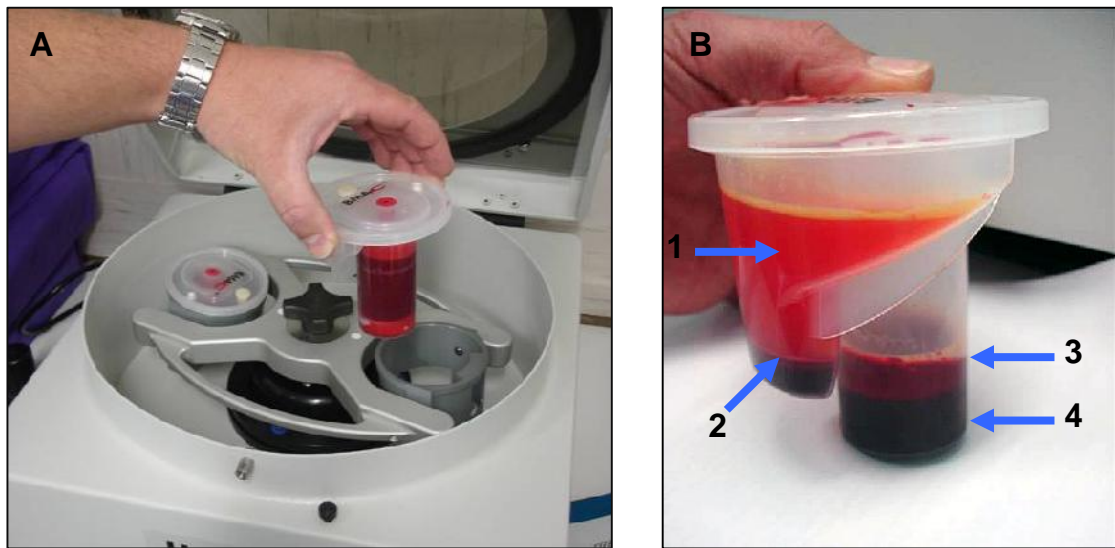
Fig 4:4 A: HARVEST centrifuge with patented bone marrow pots filled with the bone marrow aspirate. B: Post centrifuge the concentrated fraction containing the SSC is segregated within a separate compartment. 1 = Plasma, 2 = Concentrated mononuclear cell layer. 3 = floating plastic shelf designed to separate off cell fraction. 4 = waste red blood cells.

Once the SSC containing fraction of the bone marrow aspirate had been separated out by the centrifuge, the excess plasma was then aspirated off leaving a few ml. this was then used to re-suspend the buffy coat layer which was then drawn off and transferred back to the operative field. At this stage the milled allograft can be seeded with the concentrated sample and was allowed to stand with frequent agitation whilst the surgery was performed.

4:4:4 Surgical technique

Under image control a guide wire was passed into the affected area, for the cases of AVN prior MRI scans of the hip had localised the necrotic area involved. Once the surgeon was satisfied the guide wire was appropriately placed, a 12mm cannulated drill was passed up the guide wire under Image control. The drill was advanced until it rested just under the subchondral bone thus achieving initial core decompression. Any further necrotic bone proximally could then be curetted out.

The seeded allograft was then compacted using a 12mm Xchange core impactor (Stryker, Newbury UK). The impactor containing the allograft was then advanced up the canal under the subchondral bone and impacted in place with great care taken not to 'blow out' the thin overlying cortex. This process was then repeated until the drilled out channel was filled with a solid core of impacted allograft and concentrated SSC.

A similar technique was used for the fracture non union with the guide wire passed across the fracture line and the sclerotic bone ends over drilled and packed with IBG as above.
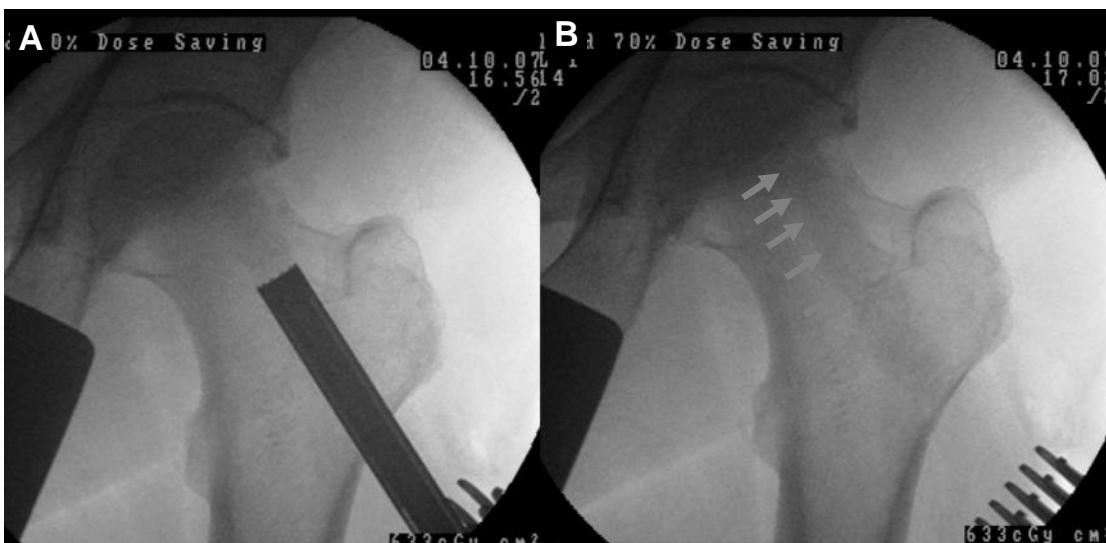
Fig4:6 A: core decompression being progressively filled up with the sample of concentrated marrow and allograft. B: final result with arrows highlighting new dense core of impacted allograft and concentrated BMA.

4:5 Parallel In Vitro Methods

Any waste allograft and bone marrow aspirate was collected with the appropriate consent and local ethics committee approval. Unfortunately no waste concentrated sample was available for one of the AVN cases so comparative analysis was not available.

4:5:1 CFU-F Count

A CFU-F is defined as a colony of cells derived from a single progenitor cell and can be used to determine the concentration of SSC within the bone marrow aspirate.

Equal volumes (0.5ml) of concentrated (BMAC) and unconcentrated (BM) bone marrow were plated straight into culture flasks to allow comparative CFU-F counts to be made.

At 2 weeks the culture plates were fixed with 95% Ethanol and an Alkaline Phosphatase stain with Napthos AX-MX and Fast violet B Salts was performed.

4:5:2 qPCR analysis

Further Tissue culture flasks were cultured to near confluence and then fixed and processed for quantative PCR analysis for Type 1 Collagen, Osteocalcin and Alkaline Phosphatase gene expression. This analysis was kindly performed by Miss S Inglis.

4:5:3 Cell Viability

Spare Allograft was seeded with both the BMAC and BM samples and impacted and contained within capsules and cultured in basal media (-MEM + 10% FCS). The samples were cultured for up to 6 weeks dependant on the quantity of the waste allograft and BM / BMAC available.

Samples were analysed for cell viability at 0, 2, 4 and six weeks using a live / dead stain of Cell tracker green and Ethidium Homodimer (Sigma Aldrich Ltd, Gillingham) as previously described.

4:5:5 Histology

Samples from each group was analysed histologically. The allogaft underwent decalcification in 0.1% TRIS / 5% EDTA solution and complete decalcification was confirmed by faxitron xray imaging. Samples were sectioned and mounted in standard fashion. Slides were stained for collagen and extracellular matrix production using Alcian Blue and Sirius Red (A+S), and to establish osteogenic differentiation of the cells they also underwent immunohistochemical staining for collagen 1 and bone sialoprotein (BSP).

4:5:6 Biochemistry

Four capsules were retained for biochemical analysis of DNA content (as a measure of cell number), and specific alkaline phosphatase activity (as a measure of osteogenic differentiation). Cells were removed from the allograft using trypsin, collagenase and multiple vortexing. Allograft was discarded, cells were fixed in ethanol and cell lysates were obtained via x3 freeze thaw cycles in Triton X. ALP and DNA assays were then performed in a standard manner with 10μl of lysate run in triplicate for each disc on a plate against x2 standards and read on a Bio-Tek KC4 and FLX-800 microplate fluorescent reader (Bio-Tek, USA).

4:6 Clinical Results

4:6:1 Fracture Non-union

The one case of fracture non union has now clinicaly and radiologicaly united and has been discharged from further follow up after over 2 years with an un united fracture prior to biological intervention with concentrated SSC grafting. Her pain control and mobility have improved as a result. There has been no signs of failure of the metalwork.

Fig (4:6) Plain Radiographs showing A: Before and B: after BMAC seeded IBG showing on the left the initial fracture line clearly visible , and on the right good callus formation with bridging bone.

4:6:2 AVN Cases

To date 3 of the cases have remained asymptomatic with no radiological signs of femoral head collapse at 25 – 47 month follow up. Patient 4 progressed on to collapse both femoral heads and has subsequently undergone bilateral THRs. Histological and mechanical testing carried out by Mr A Aarvold on the retrieved femoral heads has shown a dense central channel of bone with trabeculae crossing into the sample consistent with bone remodelling. Micro

CT analysis of the bone has shown a density profile between that of trabecular and cortical bone.

Fig 4:7 AP and Lateral Radiographs showing Rt THR performed for advanced collapse. Left hip asymptomatic, grade II AVN treated with Concentrated SSC and allograft with dense channel of IBG just visible in the superior head and neck.

Fig 4:8 Plain radiograph showing AP view of Bilateral core decompression and SSC seeded IBG

4:7 Results of Parallel In Vitro Analysis

4:7:1 CFU-F
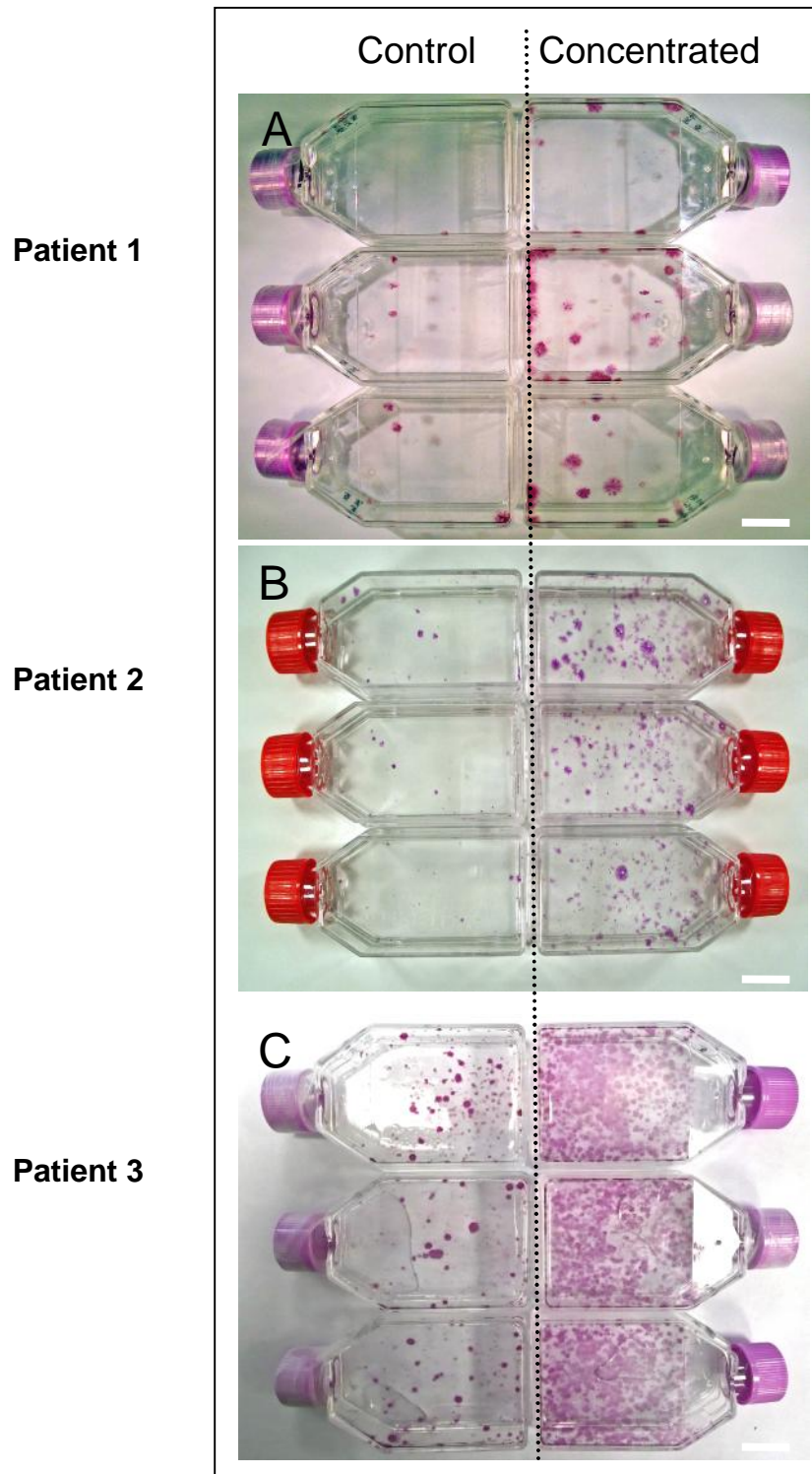
Fig 4:9 Alk Phos staining of CFU-F on T25 tissue culture flasks following 2/52 culture. Control BM samples on the left of each photo with concentrated / BMAC on the right.. A; Patient 1. B: Patient 2. C: Patient 3. Scale bars 20mm

There was significant variability in the SSC yield from the bone marrow aspirate between each patient as can be seen in figure 4:9. There are a number of factors that affect the SSC yield including patient age and co morbidities, and also the technique of obtaining the aspirate. The best yield from this small study occurred in patient 3 who was the youngest of the cases with no underlying conditions or obvious cause for his AVN. All samples did however undergo significant concentration of the SSC fraction using the centrifuge device. The CFU-F in the unconcentrated group are sparse, particularly in patients 1 and 2 (A&B Fig 4:9) and considering the large surface area of tissue culture plastic (TCP) compared to the individual allograft chips, minimal seeding would have occurred on the allograft. The concentrated fractions show marked increase in coverage of the TCP, particularly in patient 3:
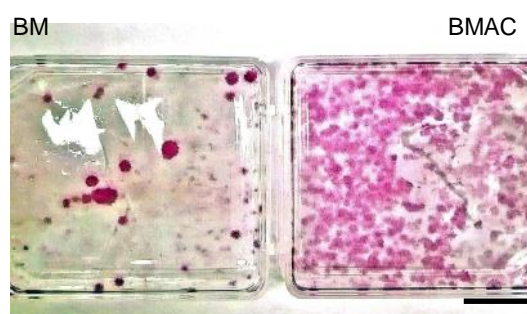
Fig 4:10 Photomicrograph of T25 Flask following Alk Phos staining of CFU-Fs with un concentrated sample BM on the left and Concentrated BMAC on the right. Scale bar 20mm

The CFU-F counts were as follows:

Patient	BM Sample (Number +/- SD)	BMAC Sample (Number +/- SD)	p value
1	4.66 +/- 1.2	61 +/-12.1	0.01
2	21.3 +/- 3.5	143 +/- 18.5	<0.01
3	87.3 +/- 16.5	412 +/- 40.1	<0.01

Table 4:1 CFU-F counts for BM and BMAC samples in all 3 patients showing significantly increased counts

Physical counting of the individual number of CFU-Fs was performed using a grid background. Table 4:1 shows that all three patient samples which were taken straight from the theatre setting and plated up in the labs without further manipulation show a highly significant increase in CFU-F following centrifuge separation with p = / <0.01.

4:7:2 Biochemistry

Four seeded allograft samples from each group were analysed for DNA content and Alk Phos Specific activity. Plain allograft samples were also analysed and the values obtained for the allograft subtracted from the BM / BMAC totals as this was considered the 'background noise' from retained cells within the allograft and not subjected to the experimental variables. Of note these background values (not included in the graph below, but subtracted from the total values for BM and BMAC) declined over the 4 week culture period presumably due to the repeated washing during media changes.

The graph below (Fig 4:11) demonstrates that there was an increase in DNA content over the 4 week culture period confirming on going cell division with increase in both concentrated and in control samples.
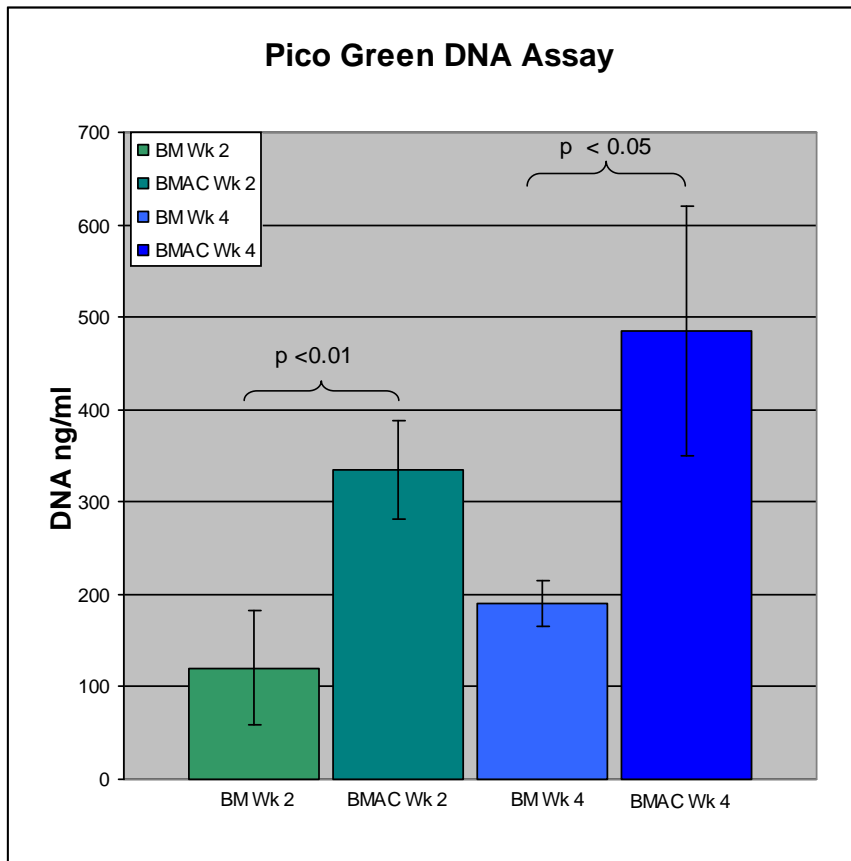
Fig 4:11 DNA content as measured by Pico Green assay. Control BM and Concentrated BMAC samples at 2 and 4 week culture. Error bars denote 1SD. P values as shown.

The mean amount of DNA at both 2 weeks and 4 weeks was significantly greater in the concentrated BMAc group compared to standard bone marrow aspirate as the control with $p < 0.01$ and $p < 0.05$ at 2 and 4 weeks respectively.
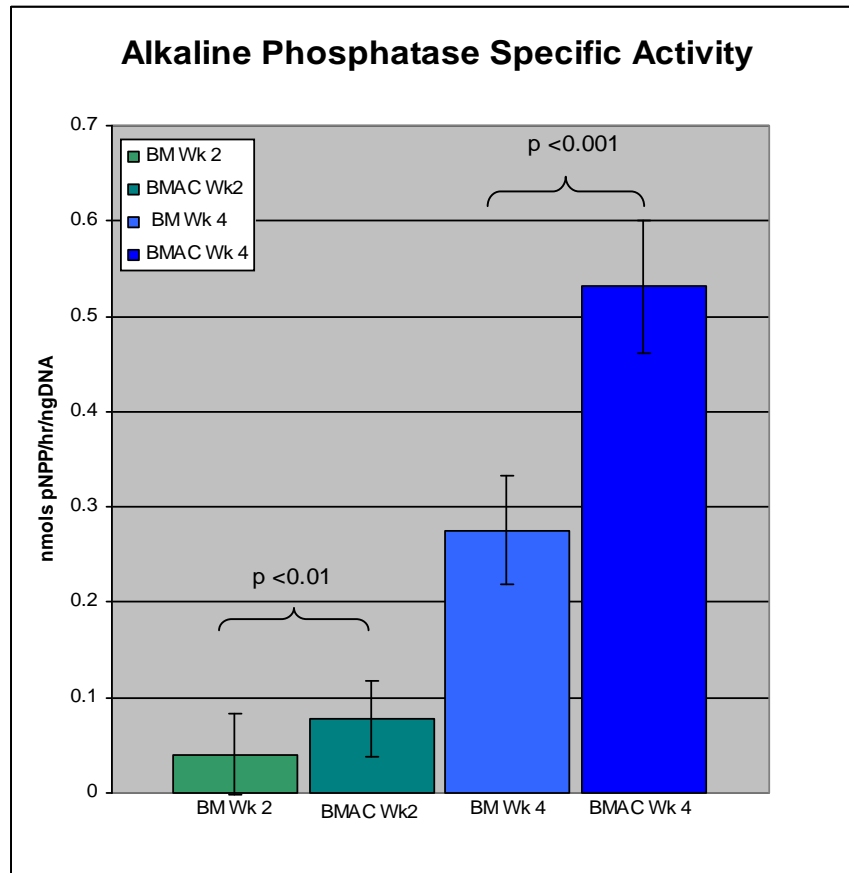
Fig 4:12: Alkaline Phosphatase Specific Activity for control and concentrated samples cultured on Allograft for 2 and 4 weeks. Error bars denote SD. P values as documented.

Figure 4:12 demonstrates an increase in the levels of Alkaline phosphatise activity over the 4 week culture period indicative of ongoing osteogenic differentiation of the samples. Comparing the unconcentrated control and concentrated group shows a highly significant difference between the 2 with $p<0.01$ and $p<0.001$ at 2 and 4 weeks respectively.

4:7:3 Quantative PCR

Below are graphical representations of the qPCR results for Patients 1 and 3. Unfortunately the sample for Patient 2 was contaminated during processing and no spare samples due to the limited supply of BMAC / BM was available. The DNA sample for Patient 1 unconcentrated was also very small with only sufficient amount for Col 1 and Osteocalcin analysis, but as Alk Phos activity had been measured via quantitive biochemical assays it was felt the other two markers for osteogenic differentiation were more important.
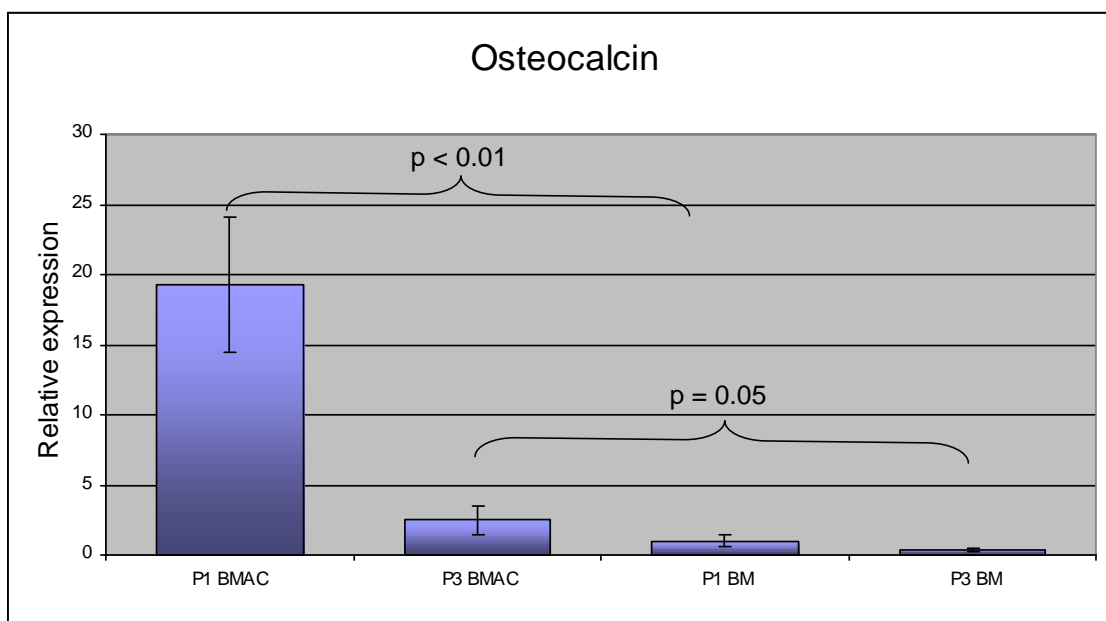
Fig 4:13 Quantative PCR Graph showing the relative expression of the Osteocalcin gene as a marker for osteogenic differentiation of the SSC all normalised to   Actin. P1 – Patient 1, P3 = Patient 3, BMAC = Concentrated group, BM = unconcentrated
Mean values n=3 with duplicate runs for each. SD for error bars

Osteocalcin is a bone-specific protein synthesized by osteoblasts that represents a good marker for osteogenic maturation. The graph in Fig 4:13 shows that the relative expression of Osteocalcin was significantly higher ($p<0.01$) in the concentrated group for patient 1 compared to the control and approaching significance for patient 3.
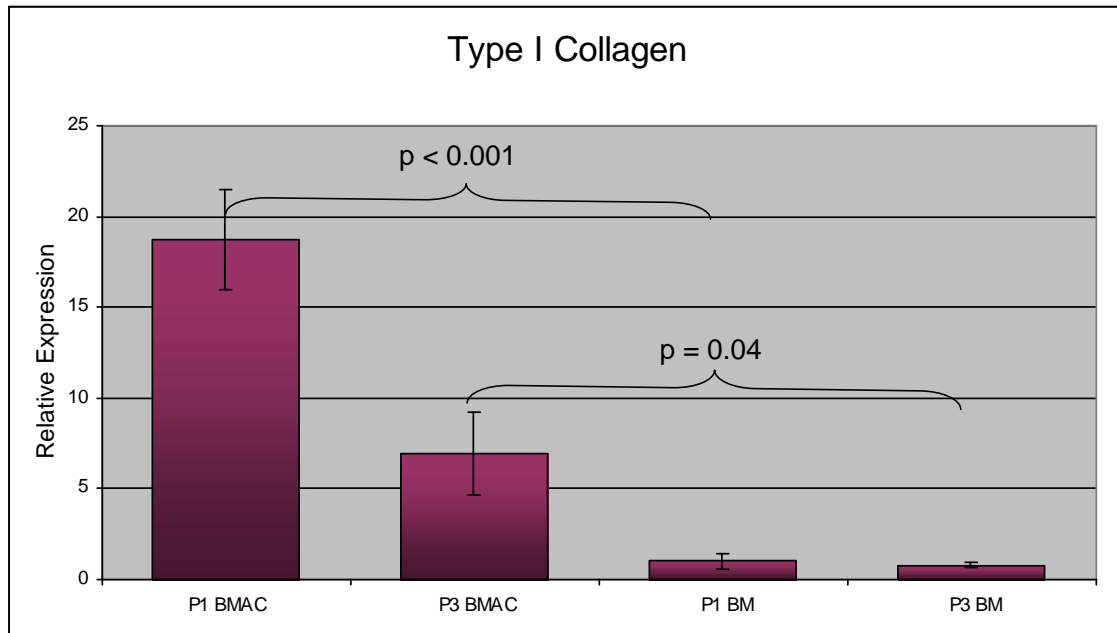
Fig 4:14 Quantative PCR Graph showing the relative expression of Type I Collagen gene as a marker for osteogenic differentiation of the SSC all normalised to   Actin. P1 – Patient 1, P3 = Patient 3, BMAC = Concentrated group, BM = unconcentrated
Mean values n=3 with duplicate runs for each. SD for error bars

Relative expression of Type 1 Collagen was also significantly increased by the concentration process, this time for both Patient 1 and 3 with $p<0.001$ and $p<0.05$ respectivly.
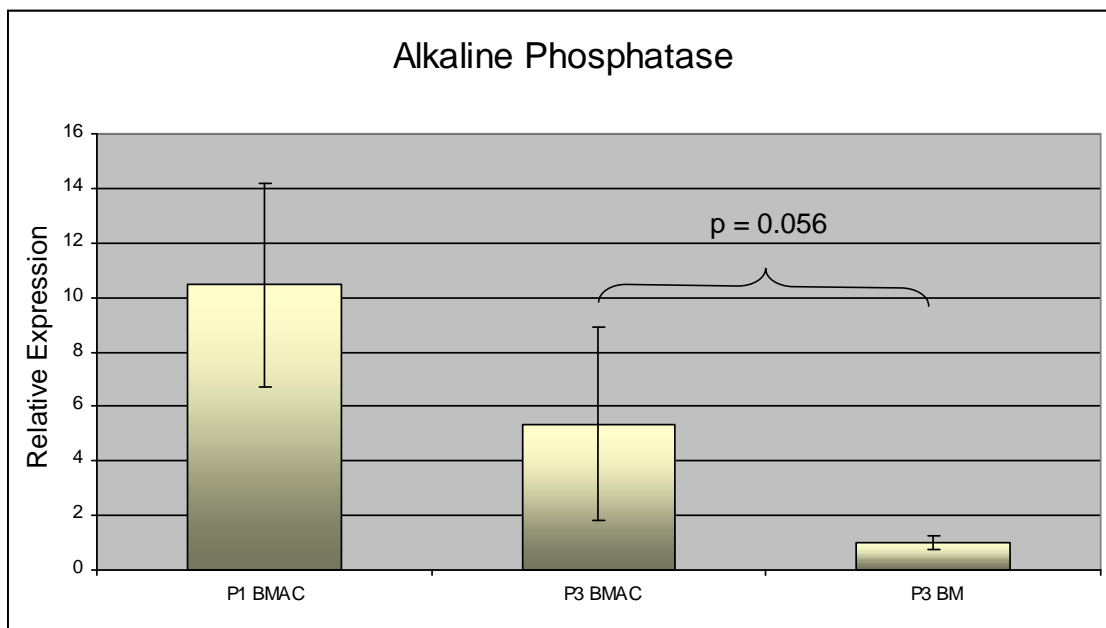
Fig 4:15 Quantative PCR Graph showing the relative expression of the Alkaline Phosphatase gene as a marker for osteogenic differentiation of the SSC all normalised to Actin. P1 – Patient 1, P3 = Patient 3, BMAC = Concentrated group, BM = unconcentrated. Mean values n=3 with duplicate runs for each. SD for error bars

As discussed earlier, sadly there was insufficient quantity of sample for the control group for patient 1 but, given the observed differences in the other biochemical assays this may have shown a difference, and when compared to the relative values of patient 3 as shown in Fig 4:15 there was a comparatively high relative expression rate. The sample from Patient 3 showed higher levels that were approaching significance, but due to the large error bars this was not statistically significant.

4:7:4 Live / Dead Staining

At set intervals during parallel in vitro culture of the samples (5, 14, 28 and 42 Days) the samples were stained with fluorescent markers to demonstrate cell viability and necrosis. Fig 4:16 Shows examples of the control and concentrated samples showing fairly sparse seeding initially, particularly in the control group. However by day 28 there was significant coverage of the allograft surface with a layer of viable SSC

BM Samples　　**Length of Culture**　　**BMAC Samples**

Day 5

Day 14

Day 28

Day 42
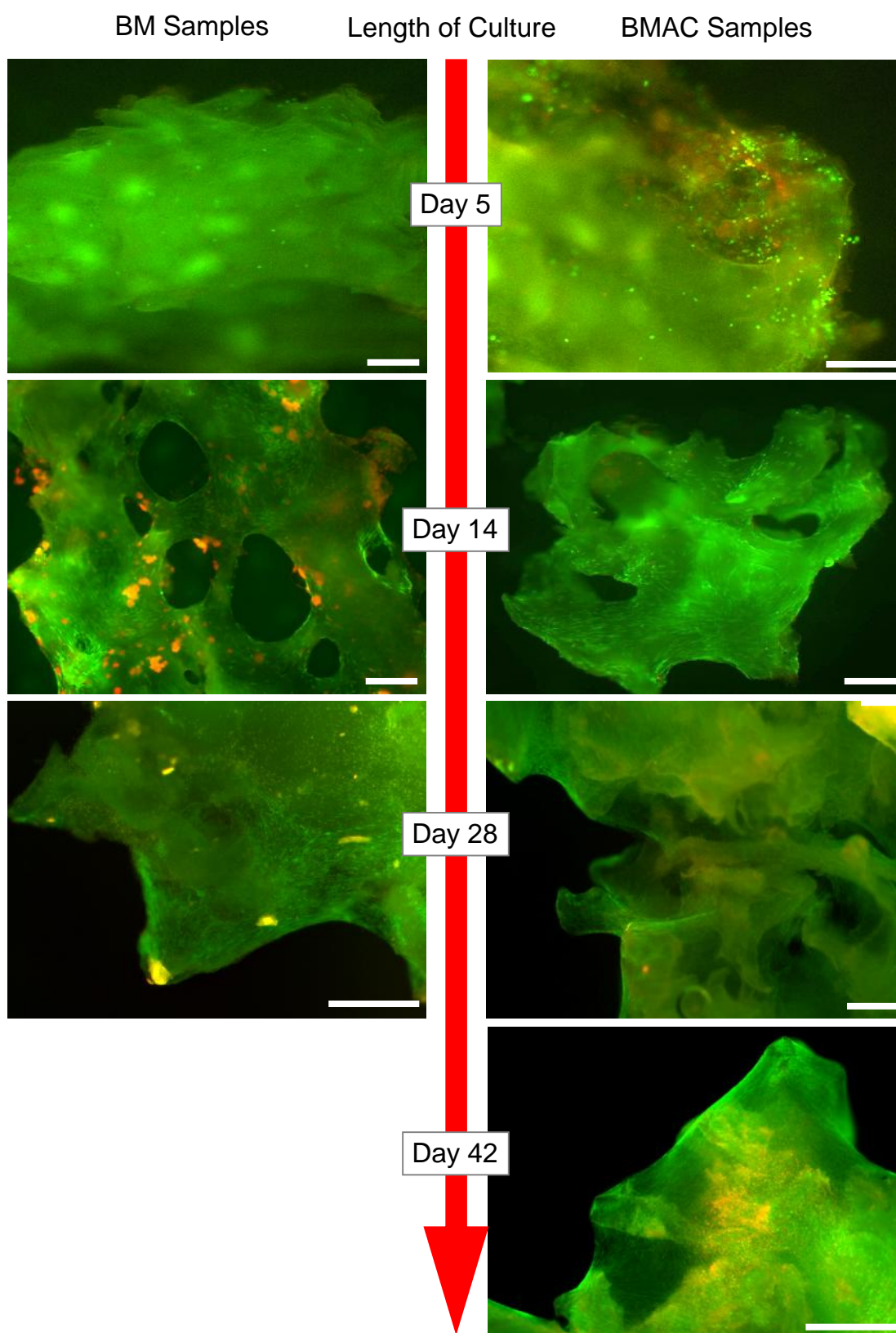
Fig 4:16 Flourescent microscopy images of Live / Dead fluorescent staining of seeded allograft at 5, 14, 28 and 42 days (Due to a finite quantity of the waste allograft available following the clinical case, no further BM samples were available for analysis at 42days). Viable / live cells green, dead cells red. Scale bar 200µm

At Day 28 in the concentrated group the cells and matrix are demonstrated bridge the gap between the allograft particles which was less evident in the unconcentrated samples at this stage. By Day 42 the spiculated form of the allograft chip has been almost completely covered and smoothed off by the abundant cellular and matrix proliferation. Fig 4:17 Shows this in more detail with the Allograft now artificially highlighted by blue we can see the initial bare allograft chip in figure A, which by week 6 in figure B has now become covered in viable SSC with bridging cells and matrix now bonding the separate fragments together.
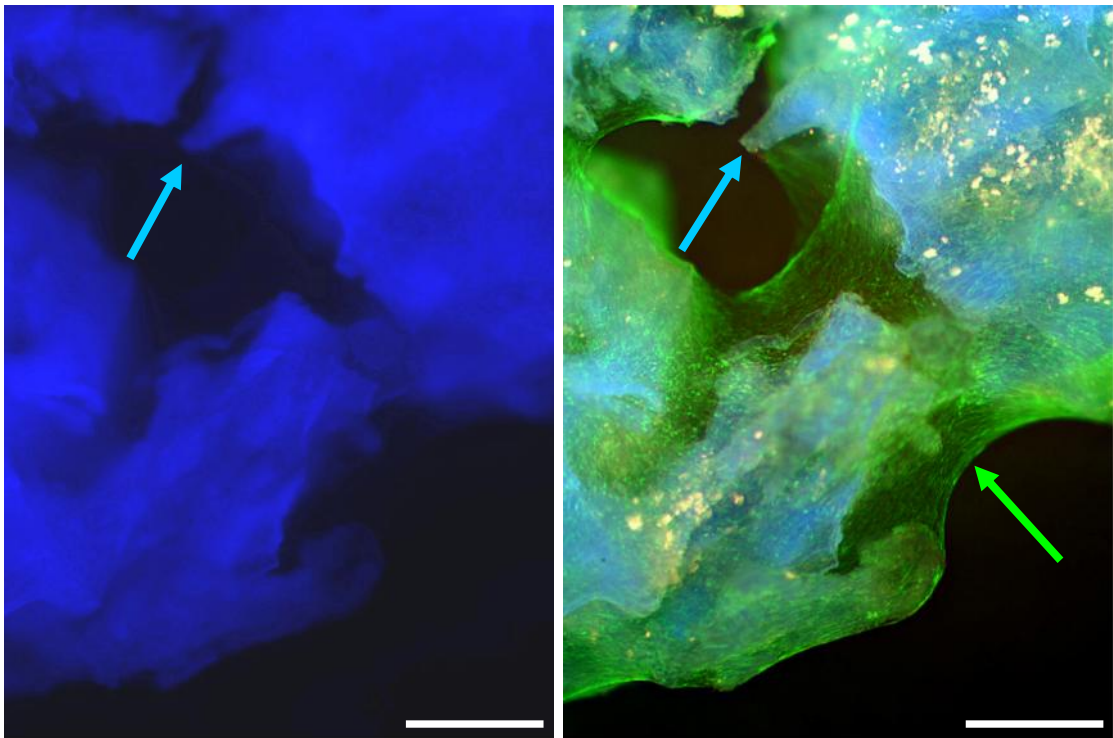
Fig 4:17 Further Live dead fluorescent microscopy images at 6 weeks culture (BMAC Group only) with the cellular layer (green arrow) now enveloping the allograft (blue arrow) and bridging the gaps. A: Representation of the bare allograft B: Allograft and SSC. Scale bar 100μm

On a number of BM samples, particularly the early ones, large collections of aggregated dead red blood cells are noted. These had a tendency to prevent SSC proliferation in that area, although as the culture progressed with multiple media changes these clumps of dead cells were washed off.

From the microscopy images we can see the initial SSC becoming adherent to the allograft, and then progressively covering the surface and finally bridging the gaps between trabeculae and other allograft particles.

4:7:5 Histology

Representative histological samples of the allograft seeded with the concentrated BMAC and un concentrated bone marrow are shown in Fig 4:18 at 2 weeks and Fig 4:19 at 4 weeks. Comparing to the plain allograft (which is the current gold standard in IBG) there is significantly more matrix formation and Type 1 collagen differentiation. Comparing the BM and BMAC samples the differences are less clear cut, but there does appear to be increased matrix (signified by the blue staining on the A&S run). On the immunohistochemical slides again the differences are not marked, but there does appear to be greater area of Type 1 collagen signified by the brown staining. The Goldners Trichrome slides, looking for new osteoid formation may demonstrate a little increase in new bone at 2weeks although there do not appear to be any significant differences at the 4 week stage.
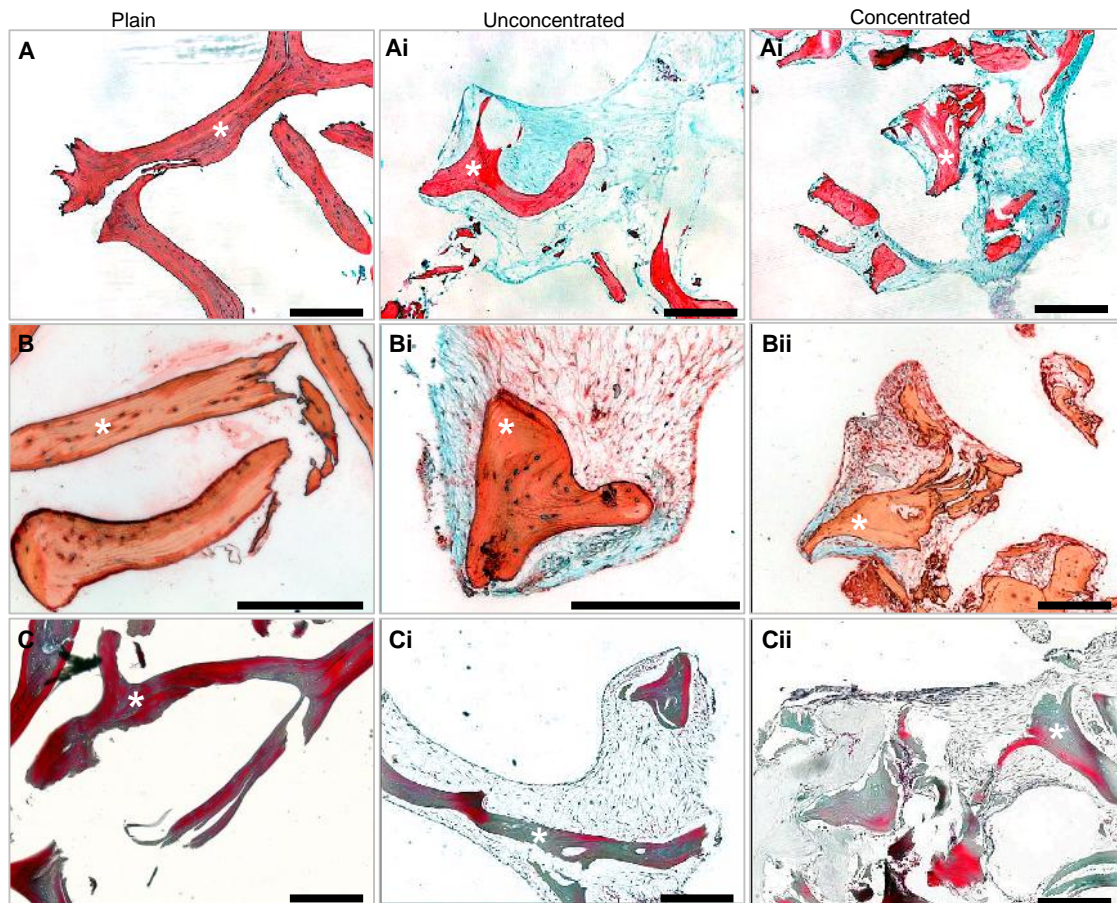
Fig 4:18 Microscopy images of Histological slides at 2 weeks culture with allograft marked by: *. Scale bars 200µm

A-Aii A&S Staining of plain allograft, BM and BMAC samples showing no cell or matrix for the control plain allograft but widespread matrix production (Blue) in both SSC seeded groups, but more pronounced in the BMAC sample.

B-Bii Type 1 Collagen staining of plain allograft, BM and BMAC samples. Brown colour denotes Type 1 Collagen with slightly more staining in the BMAC compared to BM

C-Cii Goldners Trichorome stained samples of allograft, BM and BMAC. No significant new mineralised bone was observed.
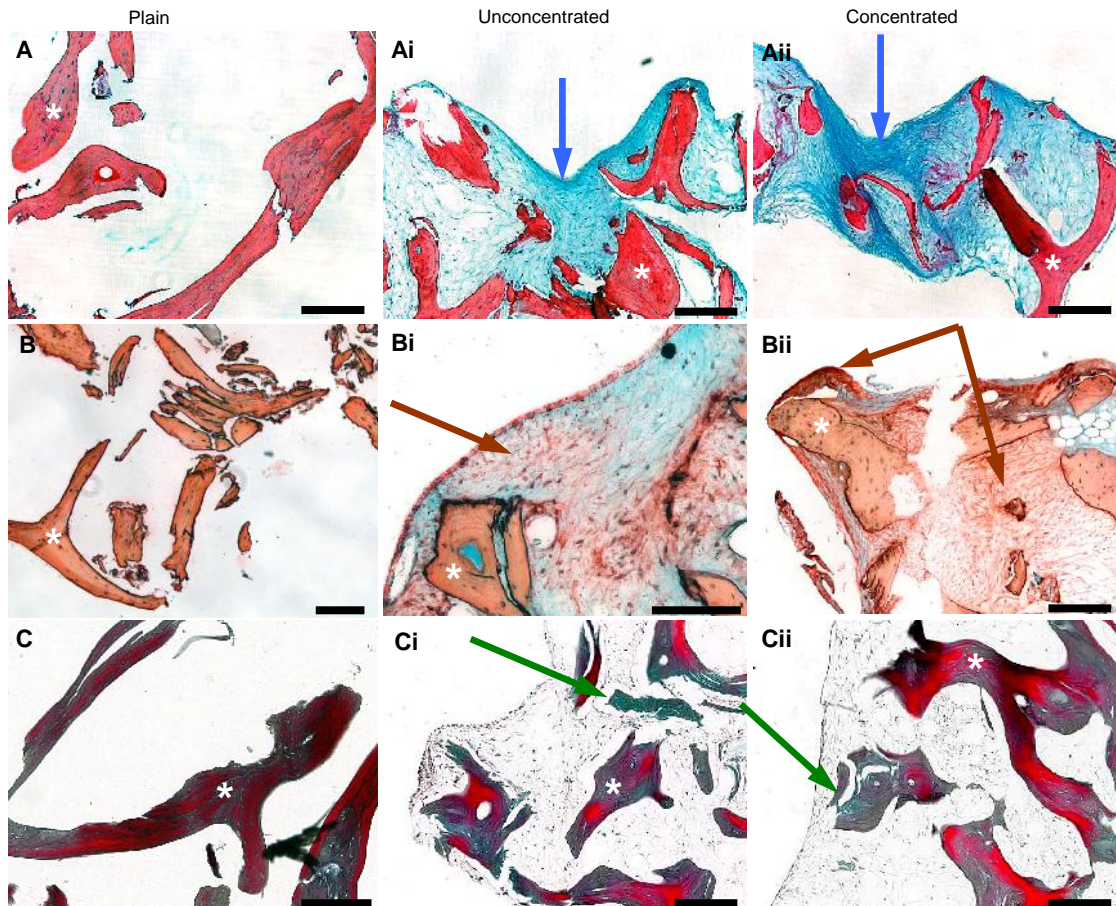
Fig 4:19 Microscopy images of Histological slides at 4 weeks culture with allograft marked by: *. Scale bars 200μm

A-Aii A&S Staining of plain allograft, unconcentrated BM and concentrated BMAC samples showing no cell or matrix for the control plain allograft but widespread matrix production (Blue arrow) in both SSC seeded groups, at 4 weeks the increased matrix in the BMAc group is more evident.

B-Bii Type 1 Collagen staining of plain allograft, BM and BMAC samples.. In the BM group some of the matrix has stained for type 1 Collagen, compared to the concentrated group where thick densly stained areas for type 1 Collagen are seen (Brown Arrows)

C-Cii Goldners Trichorome stained samples of allograft, BM and BMAC. Small areas of mineralised bone are starting to form in both samples (Green Arrows).

4:8 Discussion

Although only one case of fracture non union was treated during this study period, we have demonstrated that the bone marrow sample has undergone significant concentration of the SSC fraction, and when used in combination with IBG has gone on to unite finally. Although no control / unconcentrated sample was used clinically, parallel in vitro analysis has shown improved osteogenic differentiation. This supports the findings of Hernigou (Hernigou, Poignard et al. 2005).

The treatment of AVN remains a challenging clinical problem and despite a number of surgical treatment options any failure can quickly progress to loss of head sphericity and subsequent degenerative changes. Whilst conversion to a Total hip replacement remains the ultimate option should this occur, these patients are often young and preservation of the native hip should be the long term goal of future therapies. Core decompression +/- impaction grafting has been the treatment of choice for AVN for some years; however both methods require new bone formation and ultimately replacement of the IBG by creeping substitution. It has been shown however that in cases of AVN there is a significant reduction in number of osteoprogenitor cells, and the disease process on a cellular level can extend below the lesser trochanter (Hernigou et al. 1999; Calder et al. 2001). Histological retrieval analysis of IBG without the addition of SSC has shown that whilst revascularisation does occur and subsequent remodelling, there remains areas of fibrous tissue (Ling et al. 1993; van der Donk et al. 2002) which as the graft bed thickens may be more of a problem due to the distance from any new vessel formation. To address this problem, Tilley et all have described the addition of bone marrow aspirate to IBG in the treatment of AVN and femoral bone cysts (Tilley et al. 2006) with encouraging signs of bone integration and remodelling.

Whilst the numbers included in this study are clearly small, the principle of replacing a dead necrotic area of bone with a mechanically supportive construct that has the potential for angiogenesis and new bone formation appears sound. On review of the case that progressed on to femoral head collapse post bilateral IBG the extent of the disease on pre op MRI may not

have been fully appreciated. Retrieval analysis of the femoral head specimens carried out by our research group have shown the IBG and SSC had incorporated and remodelled well, but further areas of the femoral head more lateral to the treated area subsequently collapsed.

The parallel in vitro culture of the samples has allowed a detailed assessment of the potential benefits of the concentrated SSC fraction which we have demonstrated as giving significantly higher SSC yield and subsequent proliferation. The BMAC samples also showed improved osteogenic differentiation crucial for new bone formation to give a new solid bony construct that will hopefully continue to maintain the femoral head architecture for years to come.

Obtaining highly concentrated SSC within the theatre setting can be difficult to achieve. The centrifuge system used for this experiment has the advantage of being able to deliver that concentrated sample within the theatre and without the need for staged bone marrow harvest as is the case for culture expansion. The sample does however need to be passed out of the sterile field to undergo centrifugation and although the fluid remains sealed there are a number of steps where contamination could potentially occur. Although concentration of the SSC fraction has been achieved by up to 13x, it should be noted that the total number of SSC per ml was still low ranging from just below 8 to 165 CFU-F per ml pre concentration, well below the values obtained by Hernigou et al. Part of this may be attributed to the underlying disease process with lower numbers known to be a factor in AVN. Another issue is the harvesting of the bone marrow itself, because large volumes of aspirate are required (120ml) in order to get sufficient concentration and coverage of the IBG there is a risk of reducing the yield with venous blood pooling around the marrow. Blood also poses a problem during tissue culture as the high number of red blood cells tends to form a carpet on the graft or tissue culture plastic, potentially blocking adhesion of the SSC and this was noted for a number of the early CTG analysis. Strategies to reduce the rbc 'contaminate' and further enhance the SSC concentration and harvest ideally all within the sterile field of the theatre

setting are therefore likely to improve this technique further and ultimately improve the clinical outcome for a potentially debilitating condition.

4:9 Summary

Parallel in vitro assessment of the tissue engineering construct and subsequent retrieval analysis has given a unique insight into the bone regeneration in these clinical cases. The results from this study of significant SSC concentration, ongoing proliferation and osteogenic differentiation are encouraging for the treatment of AVN of the femoral head. It remains an ongoing study with further cases occurring recently.

4:10 Conclusion

Null Hypothesis

(i) The use of a centrifuge concentrated bone marrow aspirate in combination with impacted allograft does not improve the treatment of fracture non union or AVN of the hip

Due to the small number of cases in this study I cannot refute the null hypothesis, but the study is on going and further cases will allow a more definitive assessment of the outcomes compared to the current surgical practice.

(ii) Parallel in vitro culture of the samples does not demonstrate any biological advantage compared to the unconcentrated sample.

This has been shown to be false.

SECTION II

Enhancing the Osteogenic Differentiation of Skeletal Stem Cells

Chapter 5

The Effect of Type 1 Collagen on the Biomechanical properties of SSC Seeded Impacted Bone Graft

Chapter 6

The use of Hydroxyapatite nanoparticles to enhance the biomechanical properties of Impaction bone graft

Chapter 7

In Vivo Study on the biological effects of Type 1 Collagen and Hydroxyapatite on SSC IBG construct

Chapter 5

The Effect of Type 1 Collagen on the Biomechanical properties of SSC Seeded Impacted Bone Graft

5:1 Introduction

Type I collagen is a key extra-cellular matrix (ECM) protein and the major collagen of tendon and bone. It is important for both structural support within the ECM, but also provides key control of cell proliferation and differentiation (Dawson, Wahl et al. 2008).

Type 1 collagen is made up of a triple helical structure of alpha chains (2 alpha 1 and 1 alpha 2). It has been shown to improve SSC adherence to extracellular matrix proteins with a strong and rapid bond formed within 30 minutes (Salasznyk, Williams et al. 2004). Proliferation and osteogenic differentiation are also enhanced by contact with type I collagen, causing SSCs to express osteogenic genes (Mizuno and Kuboki 2001; Datta, Holtorf et al. 2005; Tsai, Kao et al. 2010) with the collagen integrin interaction an important signal for triggering osteoblastic differentiation.

Fresh frozen allograft contains a substantial proportion of type I collagen, but I postulate that the number of binding sites may be reduced or inactivated by the processing and/or preservation of the allograft. Here I hypothesise that coating the entire surface of milled allograft with a purified layer of type I collagen increases the number of active binding sites and enhance adherence and osteogenic differentiation. Whilst a number of the studies referenced have shown that collagen coated scaffold or tissue culture plastic can induce enhanced osteogenic differentiation, to my knowledge no work has been done on allograft. Morcellised allograft remains the gold standard for replacing lost bone stock in IBG and the use of collagen has FDA approval and is used in other medical fields. I propose that the combination of the two in the presence of SSC has the potential to form a new bony construct with enhanced biomechanical factors compared to plain allograft alone.

5:2 Null Hypothesis

1. Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not improve cell adhesion to the construct.

2. Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not improve the osteogenic differentiation of the cells on the construct

3. Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not enhance the mechanical properties of the construct

5:3 Aims

The aim of this study is to analyse the potential benefits of coating of allograft with type 1 Collagen in an attempt to improve osteogenic differentiation and the constructs biomechanical properties.

5:4 Materials & Methods

5:4:1 Type 1 Collagen

A sterile solution of Rat tail type 1 collagen (Sigma Aldrich) was used in this study. The Type I collagen was diluted with PBS to 0.25 mg/ ml. Aliquots of 6 ml were applied to 10 cm^3 portions of milled allograft, which ensured graft coverage and produced a final type I collagen load of 1.5 mg on 10 cm^3 of milled allograft. These were left for 2 hours to allow adsorption onto the allograft surface, according to manufacturer's instructions. The allograft was then left to dry under UV light and washed with PBS prior to seeding with SSCs.

Fig 5:1 Coated allograft contained in sterile pots and allowed to air dry under uv light

5:4:2 Allograft

Prepared as previously described in section 2:1. Briefly, fresh frozen femoral heads were thawed, then milled into variable sized bone chips. The chips were then washed and de fatted with hydrogen peroxide. Half the sample was then coated with Type 1 Collagen as described overleaf, with the remainder used as the plain control.

5:4:3 Cell Culture

HBMSC were harvested from consenting patients undergoing THR, and culture expanded in basal media as described in section 2:2. P1 basal culture expanded SSC were used throughout this experiment.

5:4:4 Histology

One disc from each group underwent histological analysis. The discs underwent decalcification in EDTA until completion, as measured by faxitron imaging. Slides were stained for collagen and extracellular matrix production using Alcian Blue and Sirius Red (A+S).

5:5 Study Design

The allograft was split into 2 groups with plain allograft as control and Collagen Coated as the experimental arm. In each group 15 samples were used for biomechanical analysis, 4 samples for biochemical assays and 1 sample for histological slides.

The allograft was divided into aliquots of 10cc and the graft seeded with basal cultured cells at a concentration of $5x10^4$ cells / cc . The graft was placed on an agitator within the incubator for 1 hour and then impacted with the large drop weight into the containing acrylic discs. These were then cultured in basal media for 2 weeks before undergoing mechanical testing with the custom cam shear jig, and analysis for DNA content and Alk Phos activity.
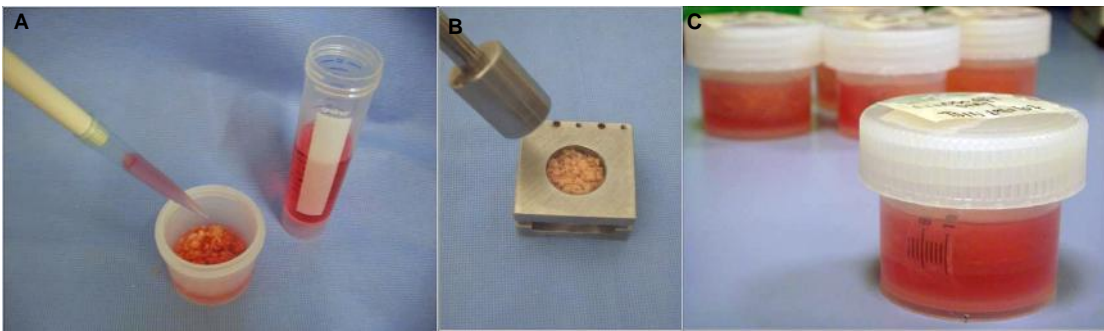
Fig 5:2 Experimental sequence. A: allograft contained within sterile pot being seeded with SSC in drip wise fashion via pipette. B: Following 1Hr of Culture in incubator samples then impacted with drop weight impactor into contained acrylic discs. C: Samples then cultured in incubator for 2 weeks covered in basal media, contained within the acrylic discs in sterile containers as shown.

5:6 Results

5:6:1 Mechanical enhancement:

Shear testing of the constructs and subsequent linear regression analysis using the Mohr Coulumb equation revealed a significant improvement in the grafts ability to resist shear and the interparticulate cohesion between the allograft chips
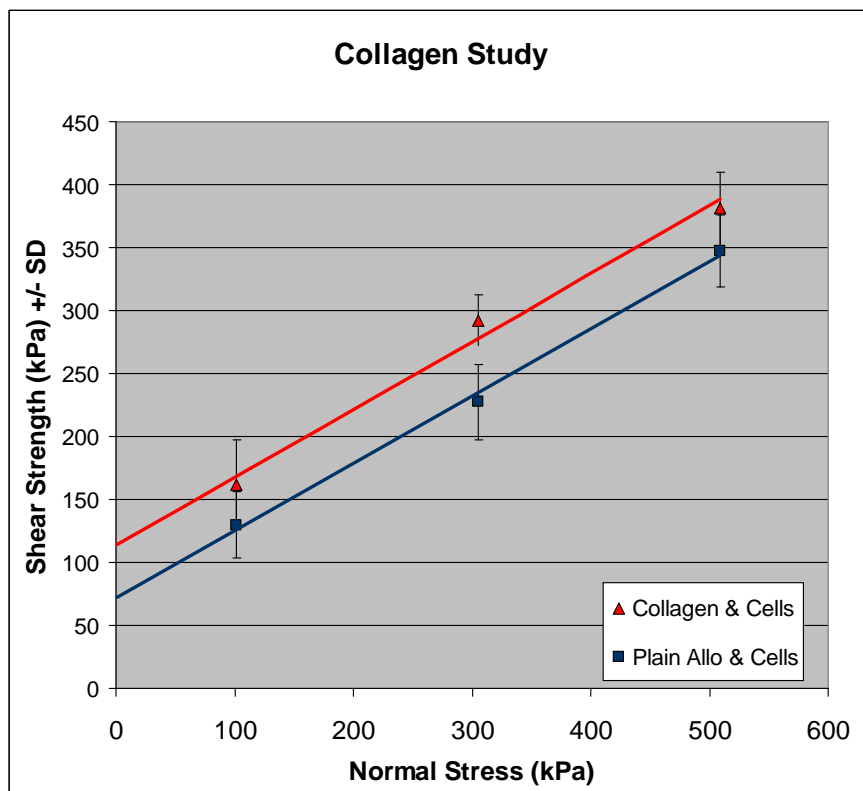
Fig 5:3 Linear regression graph demonstrating increased shear strength of collagen coated allograft over plain allograft in the presence of SSC. Lower blue line representing plain allograft and cells. Higher Red line representing Collagen coated allograft and cells. p=0.04

The linear regression analysis shown in figure 5:3 demonstrates that firstly the samples have a good correlation with linear increase and an r^2 value of >0.98. Both slopes are of a statistically similar angle which in this aggregate example signifies a similar grading to the two experimental groups of allograft. Extrapolation of the shear strength from the graph at 350kPa normal stress is shown in table 5:1 along with the interparticulate cohesion from the value at the y axis intersection.

5x10^4 Cells/cc Allograft	Shear Strength kPa	Cohesion c kPa
Basal Control	272.2	75.6
Collagen	302.3	113.3

Table 5:1 Absolute values of shear strength calculated from the linear regression analysis at a 350kPa Normal stress. Interparticulate cohesion calculated from the y axis intercept.Shear Strength and interparticulate cohesion are significantly enhanced by the addition of collagen coating. N=5

The above results show a significant increase in both the shear strength and interparticulate cohesion ($p<0.05$) following coating of the allograft with Type 1 Collagen.

5:6:2 Biochemical Analyses of IBG samples
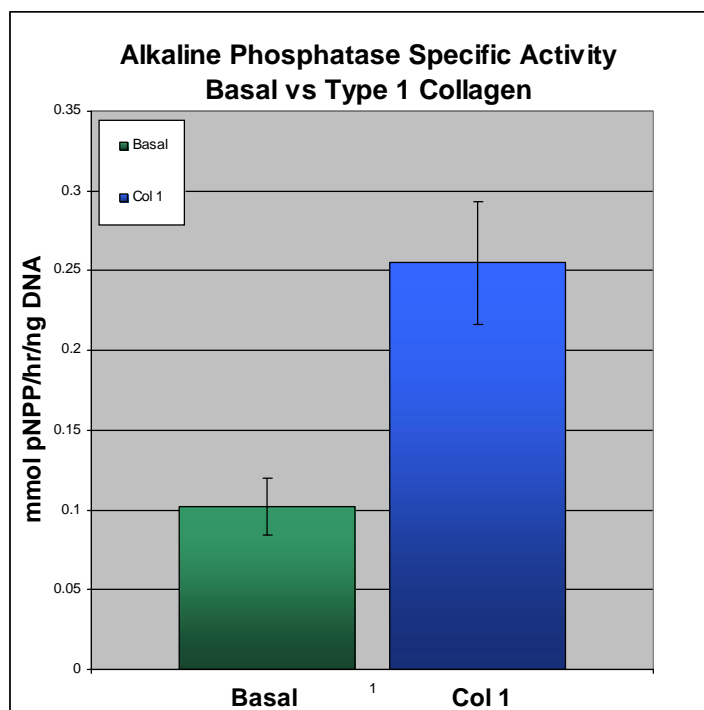
Fig 5:4 Alkaline Phosphatase specific activity for the plain allograft + SSC controls (Green) vs Collagen Coated Allograft and SSC (Blue). Mean +/- SD n=4

Fig 5:4 Shows that by the simple addition of a fresh type 1 collagen coating on the allograft bone chips, there is a significant enhancement in the osteogenic differentiation of the SSC seeded onto the scaffold 0.026+/- 0.04 vs 0.01+/- 0.02 mmolpNPP/hr/ng DNA (p<0.05).
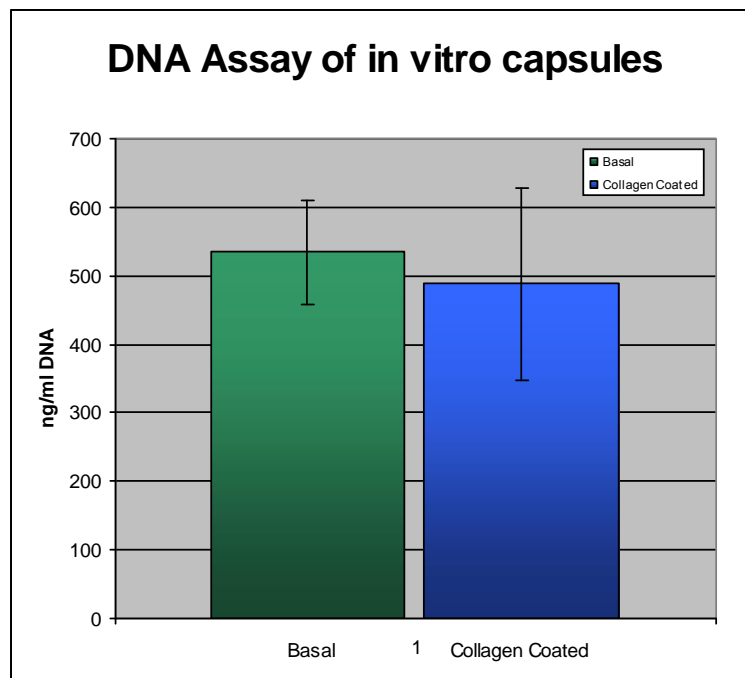
Fig 5:5 Pico green DNA assay of Plain allograft + SSC (Green) vs Collagen coated Allograft + SSC (Blue). Mean +/- SD n=4

There was however no significant difference in the DNA content of the samples. As both samples were seeded with the same number and concentration of SSC, DNA content was used as a marker for cell adherence, as although these results are following 2/52 culture, if more cells had adhered at the start of the culture process, then by the time of analysis the difference should have been even more marked with exponential expansion. Although there are other cofounding variables that may have influenced this result and analysis immediately following seeding may have been a more precise measure, the net aim was to see if there was sustained increased SSC proliferation to enhance the biomechanical properties of the graft.

In order to demonstrate a visual difference in the degree of osteogenic differentiation between the two groups to go along with the quantitive assays, half of a 6 well culture plate was coated with type 1 Collagen prior to seeding and basal culture. Alkaline Phosphatase staining was then performed , the results of which are shown below:
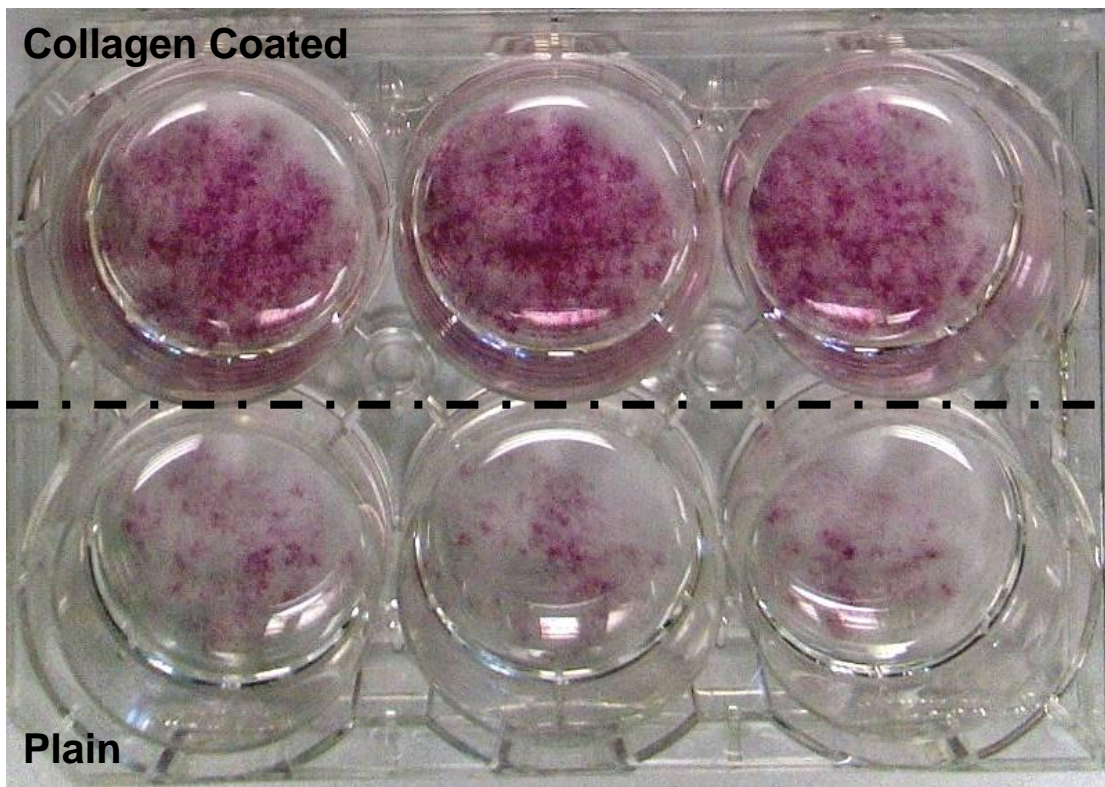
Fig 5:6 Photomicrograph showing six well plate 14 Days after seeding with equal concentrations of SSC into each well. Alkaline Phospatase staining performed to demonstrate marked increase in ALP activity in the Collagen coated group.

The marked difference in staining again strengthens the argument that type I Collagen coating can improve osteogenic differentiation.

5.7 Discussion

Type 1 Collagen is abundant within the allograft, but this study has demonstrated that by coating the allograft with a fresh layer of Collagen the biomechanical properties can be enhanced. As the cavitatory defects encountered in clinical practice increase, so the need for improved biological and mechanical function of that graft bed needs to be enhanced. Its role is to provide a solid resistance to shear in the post operative period to allow early return to weight bearing and function, and also enhance new bone formation to form a living construct that can support and remodel around the new implant. I have demonstrated that Type 1 collagen coating can significantly enhance the shear strength after 2 weeks of culture. I have also shown that Type 1 Collagen can improve the osteogenic (as depicted by the raised Alk Phos assays) differentiation of the SSC confirming similar results to previous studies(Mizuno and Kuboki 2001).

In Mizunos study they used a type 1 collagen matrix and analysed both the ALP specific activity and also the expression of gene expression of the osteogenic markers ALP , Osteocalcin, Osteopontin and Bone Sialoprotein at 1,2 and 3 weeks. They showed that at week 1 the differences between the two groups was minimal, but by 2 weeks there was significantly increased expression of ALP. Mineralisation of the samples only started to occur around the three week stage. Although no qPCR analysis was performed in this experiment, the early signs of mineralisation of the Type 1 Collagen coated samples were observed on the 6 well plates (Fig 5:7) and similar ALP results were observed in our study.
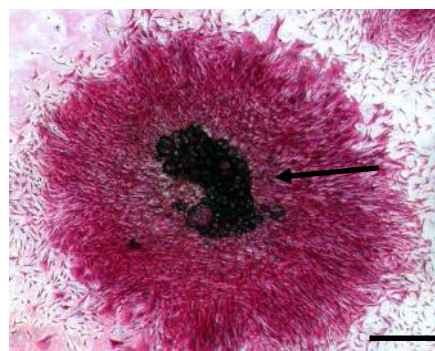
Fig 5:7 CFU-F on Collagen coated TCP at 2 weeks culture showing early central mineralisation (arrow). Scale bar 200µm

It has been shown that Type 1 collagen can form a rapid bond with SSC within 30min (Salasznyk et al. 2004) however the results from my study did not show any significant increase in DNA activity between the two groups. Measurement of DNA quantity is not a direct measurement of the SSC's rate of adhesion and there are many factors that may have affected the result. The samples were seeded and incubated for 1 hour prior to IBG as this protocol had worked for previous experiments, but it may be that this time was too long and the plain control group was able to bind with the majority of the SSC in that time. Further work should therefore be directed at analysing how short a time is required for the collagen coated allograft to bind sufficient SSC as the shortest time possible is advantageous within the theatre setting.

This study has confirmed the finding of others namely that Type 1 Collagen does appear to trigger osteogenic differentiation within the stromal cell population (Mizuno et al 2001; Salasznyk et al 2004). The exact mechanism for this is not fully understood, but Mizuno was able to demonstration that collagen a2- 1 integrin interaction was an important signal for the osteoblastic differentiation of bone marrow cells. This study has taken these results further by showing that not only does enhanced osteogenic differentiation occur, but that there is also a significant enhancement in the mechanical properties when used in the IBG setting. I postulate that whilst the quantity of cells present was not significantly different to the control, greater amounts of mineralised matrix are being produced by the enhanced osteogenic cell differentiation leading to a stronger bridging between allograft chips thus increasing the shear strength.

Direct measurement of the strength of osteoblast like cell adhesion at a cellular level has been studied by Takai et al. They observed that the strength of bond as measured by the elastic modulus of the cells to various ECM proteins including Type 1 collagen was significantly greater when compared to cells cultured on glass (Takai et al 2005).

So from the evidence from this study and the literature I conclude that Type 1 Collagen promotes rapid and strong stromal cell adherence, enhanced osteogenic differentiation and subsequent increased mineralised matrix

production which when used in vivo significantly enhances the biomechanical properties of IBG.

Translation of these techniques into the theatre setting should be relatively facile. The milling of allograft is generally done at the start of the case, and if necessary this step could be completed prior to commencing surgery. The steps themselves are straightforward and the potential clinical benefit we postulate could be significant.

5:8 Summary

Coating of allograft with type 1 collagen prior to seeding with SSC causes a significant enhancement in the osteogenic differentiation and shear strength of the IBG construct. It is a facile technique that should be readily transferable to clinical practice.

5:9 Conclusion

Null Hypothesis

- o Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not improve cell adhesion to the construct.
    - This hypothesis is *true* for our experiment

- o Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not improve the osteogenic differentiation of the cells on the construct
    - This hypothesis has been proven *False*

- o Coating of milled allograft with Type 1 Collagen prior to seeding with SSCs will not enhance the mechanical properties of the construct
    - This hypothesis has been proven False

Chapter 6

The use of Hydroxyapatite nanoparticles to enhance the biomechanical properties of Impaction bone graft

6:1 Introduction

(HA) is a naturally-occurring mineral that comprises the major inorganic constituent of bone which was first confirmed by Dejong in 1926 (Dejong 1926). Its probable first use in Orthopaedics was in crystalline powder form for the treatment of fractures by Albee, F. H., and Morrison in 1920 and shown to improve callus formation (Albee 1920). Due to its non-toxic, non-inflammatory, osteoconductive properties and its structural characteristics, it has enjoyed widespread application as a surface-coating for orthopaedic implants to promote osseous integration (Trikha et al. 2005)

Its use in modern Orthopaedics came to prominence with the coating of the Furlong hip stem which commenced in 1985 (Furlong and Osborn 1991; Furlong 1993) which continues to show exceptional clinical results to date with 99% survival at 13 years (Shetty et al. 2005).

The functionality of HA has recently been further exploited by biotechnological innovation, which has allowed the synthesis of HA nanoparticles (HAp) within a colloidal suspension (Zhou et al. 2008). This technique provides a useful method of delivery at the cellular level, putatively allowing integration between the beneficial properties of cells within impacted graft with the mechanical advantages of HA. It is thought that the concerted action of the cell and HA within bone graft will act synergistically to improve the graft's characteristics.

The enhancement of osteogenic differentiation has been shown in 3D pellet culture (Babister et al. 2009), but no study to my knowledge has analysed the biological and mechanical effects when used in combination with allograft and SSC.

6:2 Aims

This study was designed to assess the biological and mechanical enhancement of IBG allograft when seeded with SSC that have been cultured with HA nanoparticles. And to assess the practicalities of transferring these techniques into the operating theatre

6:3 Null Hypothesis

1. Coating of milled allograft with SSC cultured with HAp nanoparticles will not improve the osteogenic differentiation of the cells on the construct
2. Coating of milled allograft with SSC cultured with Hap nanoparticles will not enhance the mechanical properties of the construct

6:4 Materials and Methods

6:4:1 HA nanoparticles

Aqueous colloidal suspensions of arginine-functionalised HA nanoparticles were prepared as previously described (Gonzalez-McQuire R 2004; Babister, Hails et al. 2009). Briefly, an aqueous solution of 1M $CaCl_2$ and 2M arginine was prepared (50 ml, pH 9). This was cooled to 9°C and an aqueous solution of 0.33M Na_2HPO_4 (50 ml, pH 9) was added dropwise at a rate of 13 ml h^{-1}, with vigorous stirring. The reaction temperature and pH were maintained during the addition. After completion of the addition of the phosphate solution, the temperature was increased to 50°C. The pH was monitored and maintained at 9 for a further 1 hour. The reaction solution was then aged at 50°C, with continued vigorous stirring for a further 15 hours, resulting in a stable colloidal solution. The HA/amino acid nanoparticles were sterilised by – radiation at 2500 rads for 5 hours prior to formation of the cell/HA conjugates.

To prepare the cells treated with HAp, P1 culture expanded human SSCs were trypsinised and suspended in basal media The HAp colloid was then added to the cell suspension in universal tubes (20 ml per million cells, 7 x 10^8 particles

per cell) and incubated for 1 hour before the sample was re-spun, washed to remove any excess HAp and resuspended in media prior to seeding on the scaffold.
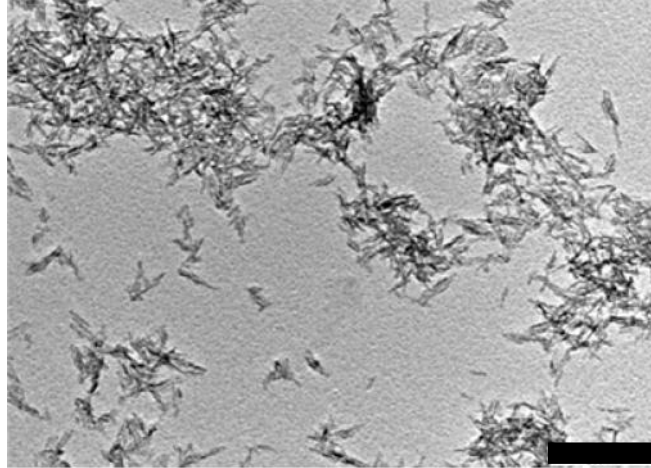
Fig 6:1 Electron microscopy image of Hydroxyapatite nanoparticles in solution. Scale Bar = 200nm

6:4:2 Allograft Seeding

SSCs were seeded onto each sample of allograft at the specific seeding density of 5×10^4 cells per cm^3 of allograft. Following trypsinisation from tissue culture flasks, cell number was determined using a haemocytometer and cells were suspended in equal aliquots of 5×10^5 cells in 6 ml basal media, this being the volume necessary to cover 10 cm^3 of milled allograft. Cell suspensions were added to sterile pots containing milled allograft and placed on an agitator within the incubator for 1 hour to optimise cell adherence prior to impaction. This period was limited to 1 hour in order to simulate time that would be available intra-operatively, thereby ensuring relevance for clinical translation.

6:4:3 Graft impaction and culture

As described in the cell concentration and Collagen studies and section 2:5:2. The larger impactor was used and a total of 15 HAp SSC + Allograft discs and 16 Controls of plain allograft and SSC were impacted and then cultured for 2 weeks in basal conditions.

6:4:4 Mechanical testing

Using the custom cam shear tester 12 control and 12 HAp IBG discs were put under 3 compressive loads and sheared at a constant rate until failure as previously described in section 2:5.

6:4:5 Biochemistry

4 Control and 4 HAp discs were retained for DNA and Alkaline phosphatise assays and processed as before as described in section 2:3.

6:4:6 Electron Microscopy

A further experimental run was performed to enable more detailed examination of the samples in collaboration with Miss L Hails. Cell harvest, expansion, Graft preparation, HAp seeding and impaction of the graft along with the 2 week culture was performed by myself, the final sample preparation and scanning was performed by Miss Hails.

Identical steps as above were taken for the preparation and culture of the samples, the only difference being the smaller impactor was used to impact into customized perforated electron microscopy pots see Fig 6:2. These were cultured in tandem with the larger samples in basal media for 2 weeks and then processed as detailed below.
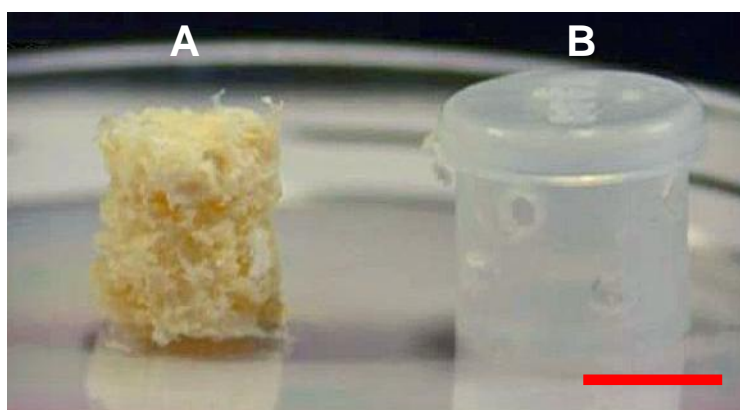
Fig 6:2 A: Allograft having been removed from the capsule, fixed and freeze dried ready for processing as below. B: Small perforated modified electron microscopy capsule used for containment during culture of the sample. Scale bar 5mm

For SEM, TEM, EDXA and ultramicrotomy, samples were fixed using 4% paraformaldehyde in PBS (pH 7.2). IBG samples for chemical and SEM analysis were fixed and freeze dried. For ultramicrotomy, samples were stained with osmium tetraoxide and subsequently embedded in low viscosity resin (Agar Scientific Ltd, Stansted, UK). 50-75 nm thin sections were prepared and observed by TEM after post-staining with lead citrate and uranyl acetate stains. For TEM imaging, a JEOL JEM 1200 EX Transmission Electron Microscope (JEOL UK Ltd, Welwyn Garden city, UK) was used. SEM analysis was performed using a JEOL JSM 6330F Scanning Electron Microscope and an Oxford Instruments ISIS 300 system (Oxford Instruments, Abingdon, UK) was used to obtain Energy Dispersive X Ray Analysis (EDXA) data of the samples.

6:4:7 Analysis by Fourier Transform Infrared (FTIR) Spectroscopy

IBG samples were fixed in 4% paraformaldehyde in PBS, washed 3 times with deionised water and freeze-dried before processing into potassium bromide (KBr) disks for analysis using a Perkin Elmer Spectrum One FTIR spectrometer (Perkin Elmer, Cambridge, UK).

6:8 Results

6:8:1 Mechanical Shear Testing

HAp seeding of the SSC prior to IBG caused a significant improvement in the grafts ability to resist shear under physiological loads. There was also a significant rise in interparticulate cohesion from 75.6 to 89.4kPa ($p<0.05$)
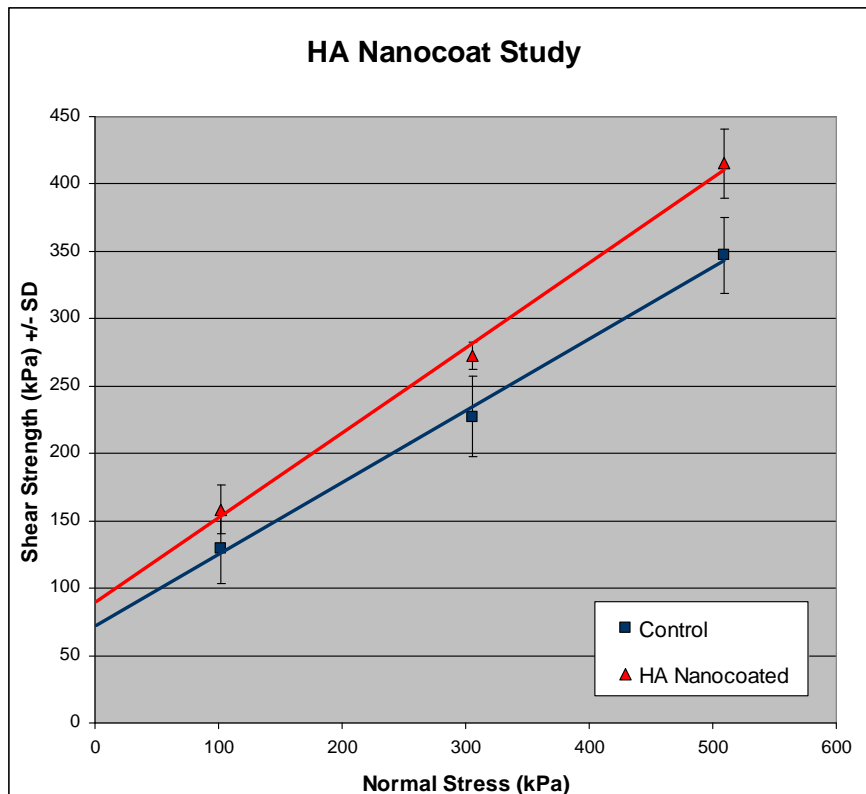
Fig 6:3 Graph demonstrating linear regression analysis of the stress strain results at 10% Strain. Allograft and SSC in blue and HAp and SSC in Red. After 2/52 culture in basal media

Linear regression analysis showed good correlation with $r^2>0.98$ confirming the aggregate obeyed the Mohr Coulumb failure law. Although the slopes of the two lines observed in Fig 6:3 look different, on further statistical analysis (ANCOVA test) there was no significant difference ($p>0.05$) implying that the composition of the graft aggregate was similar for experimental and control groups. Extrapolation of the shear strength at a physiological load of 350kPa along with the interparticulate cohesion are shown in table 6:1. Demonstrating a significant ($p<0.05$) increase in the shear strength.

5x10^4 Cells / cc Allograft	Shear Strength kPa	Cohesion c kPa
Basal Control	272.2	75.6
HAp	309.0	89.4

Table 6:1 1 Absolute values of shear strength calculated from the linear regression analysis at a 350kPa Normal stress. Interparticulate cohesion calculated from the y axis intercept. Shear Strength and interparticulate cohesion are significantly enhanced by the addition of HAp seeded SSC ($p < 0.05$) n=5

6:8:2 Biochemical analysis of cultured capsules

Biochemical analysis of the samples with Pico Green assay for DNA content revealed no significant difference between the two groups.
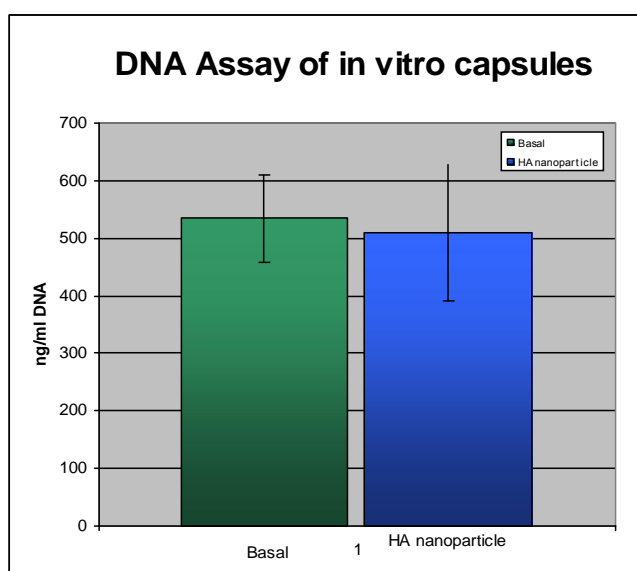
Fig 6:4 Pico green assay for DNA content in control and HAp samples. N= 4 $p > 0.05$

Alkaline phosphatise specific activity was used as a marker for the amount of osteogenic differentiation between the two groups. Fig 6:5 shows a graphical representation of the difference with the basal control at 0.10 +/- 0.02 vs HAp sample 0.18 +/- 0.03 mmol pNPP/Hr/ngDNA which was significantly different (p<0.05).

Alkaline Phosphatase Specific Activity
Basal vs HA Nanoparticle
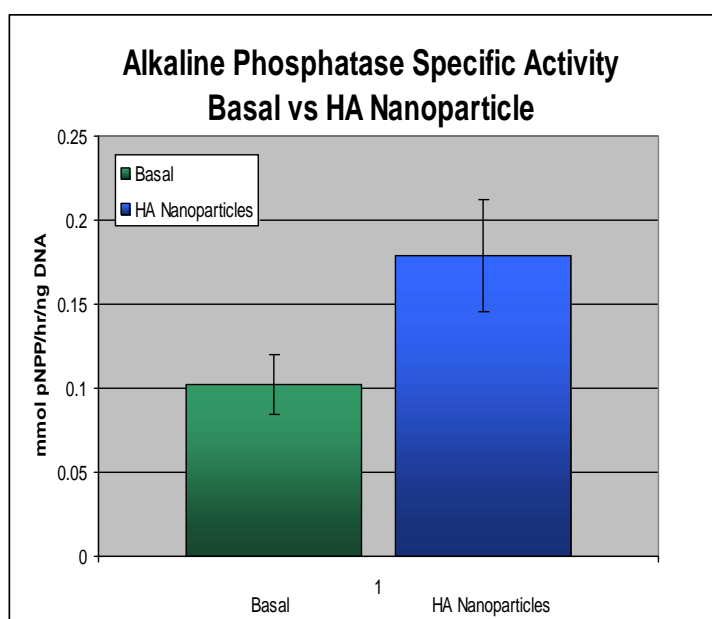
Fig 6:5 Alkaline Phosphatase specific activity for the control and HAp samples. N=4 p<0.05

6:8:3 Alkaline Phosphatase staining of monolayer culture

The biochemical assays have demonstrated a quantative difference in the osteogenic differentiation of the HAp cultured cells. In order to demonstrate a more visual representation of the difference, the two groups were also cultured in monolayer on 6 well plates, the only difference being half of the sample undergoing prior addition of the HAp as described above. After 14 Days culture, an Alkaline Phosphatase stain was then performed. A photograph of the result is shown in Fig 6:6 and 6:7 demonstrating a marked increase in ALP activity for the HAp group.
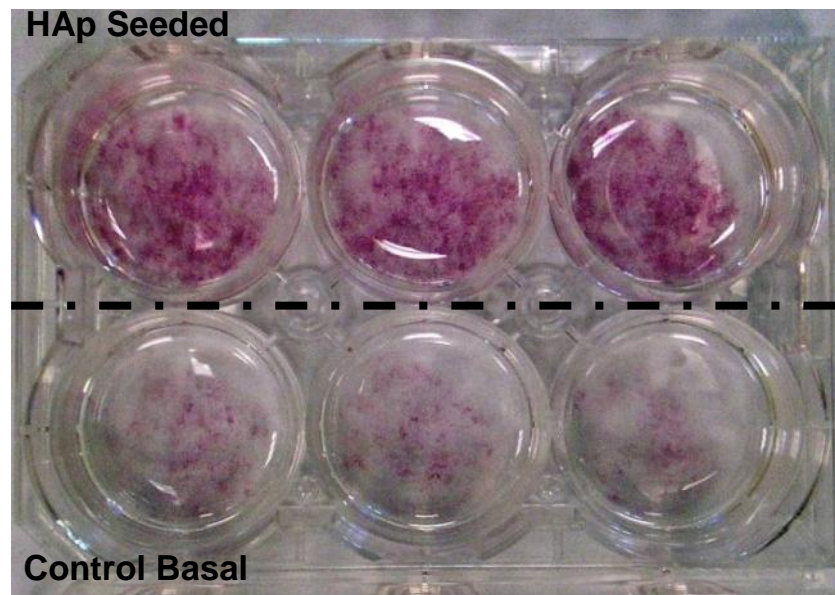
Fig 6:6 Alkaline Phosphatase stain on 6 well culture plate with half seeded with normal SSC and half with SSC cultured with HAp. Both then cultured in basal media for 2/52 Basal Control
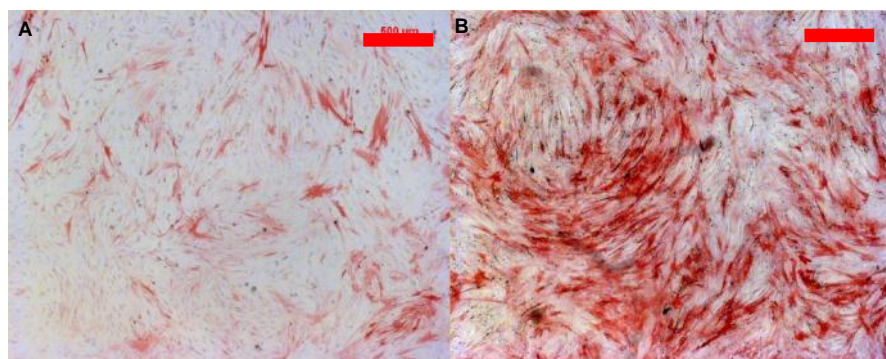
Fig 6:7 Photomicrograph of the six well plate in Fig 6:3. A: Basal control on left, B: HAp on Right showing marked increase in Alkaline Phosphatase staining. Scale Bar = 500 m

Increased early signs of matrix mineralisation were also observed in the HAp sample.

6:8:4 Electron Microscopy

TEM imaging as seen below has demonstrated stromal cells, which have incorporated HA into vesicles within the cytoplasm (Figure 6:8). Although the mechanism is not fully understood large amounts are seen within the cells with little remaining in the extracellular space.
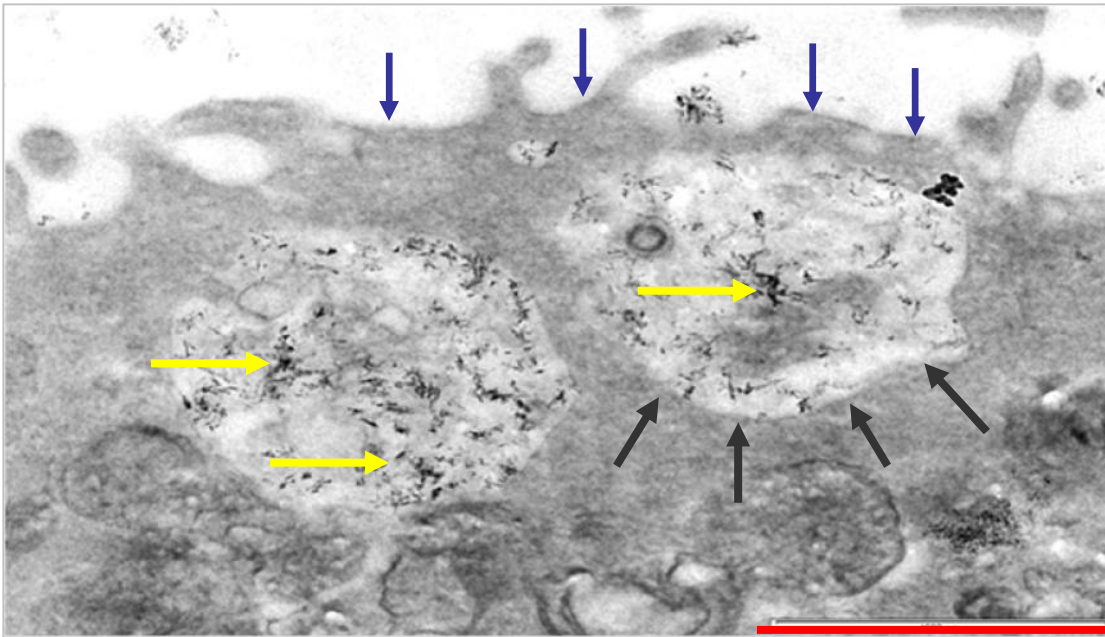
Fig 6:8 TEM image of Skeletal Stem Cell in section. Blue arrows highlight Cell membrane. Grey arrows highlight vesicle within cell containing multiple HAp nanoparticles, the small dark rods highlighted by the yellow arrows. Scale bar 1000nm

Microtomy of IBG samples after 21 days clearly identified extracellular matrix production in basal and HAp/R-treated samples (Figure 6:10 a and c respectively) and HBMSCs at the allograft chip surface (Figure 6:10 b). Areas of mineralisation, distinct from the allograft can be seen along with new matrix produced by HBMSCs in regions between adjacent allograft chips in HAp/R-treated samples (Figure 6:10 e, shown without post-stains for clarity and at higher magnification). In addition, possible mineralisation of the collagen fibres may be seen in sections of the nanoparticle treated sample (Figure 6:10 c and d, indicated by arrows).
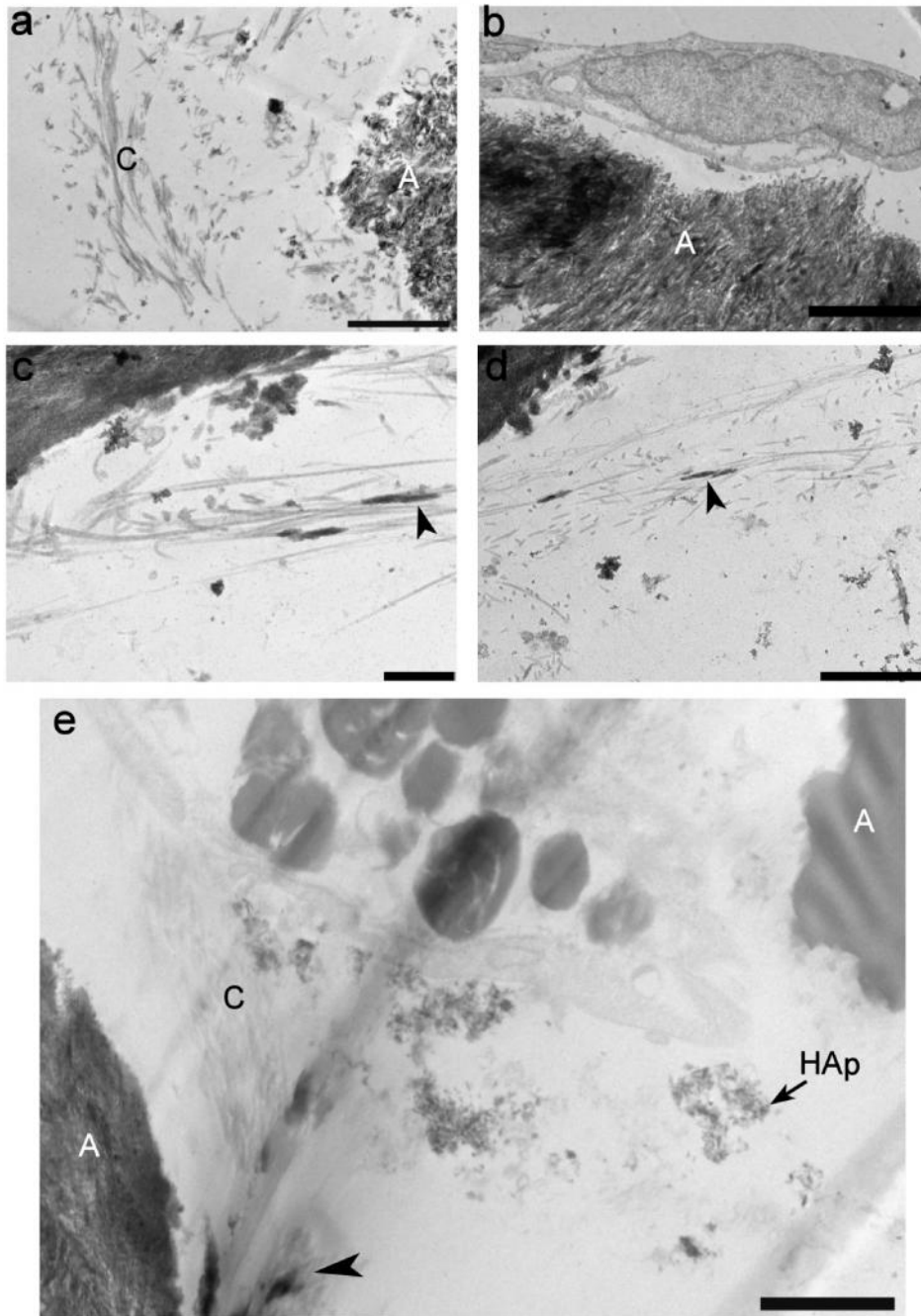
Figure 6:10 TEM images of IBG samples in cross-section stained with uranyl acetate and lead citrate:
a: Basal control, scale bar = 1 μm
b - d: HA-treated, Scale bars = 2 μm, 500 nm, 1 μm
e: HA-treated, unstained, scale bar = 1 μm.

A = allograft, C = collagen, HAp = hydroxyapatite particles, arrows demonstrate areas of mineralised collagen.

SEM analysis of the IBG samples showed a significant amount of new extracellular matrix production for samples cultured with and without HA

(Figure 6:9 ai, bi) compared with the untreated morselised allograft (Figure 6:6c).

We can see in Fig 6:9c that the surface of the plain allograft remains bare compared to the cell cultured samples where cells and matrix can be observed deposited across the surface of the bone chips and providing cohesion between the individual pieces of allograft (Figure 6:9 ai, bi and d).
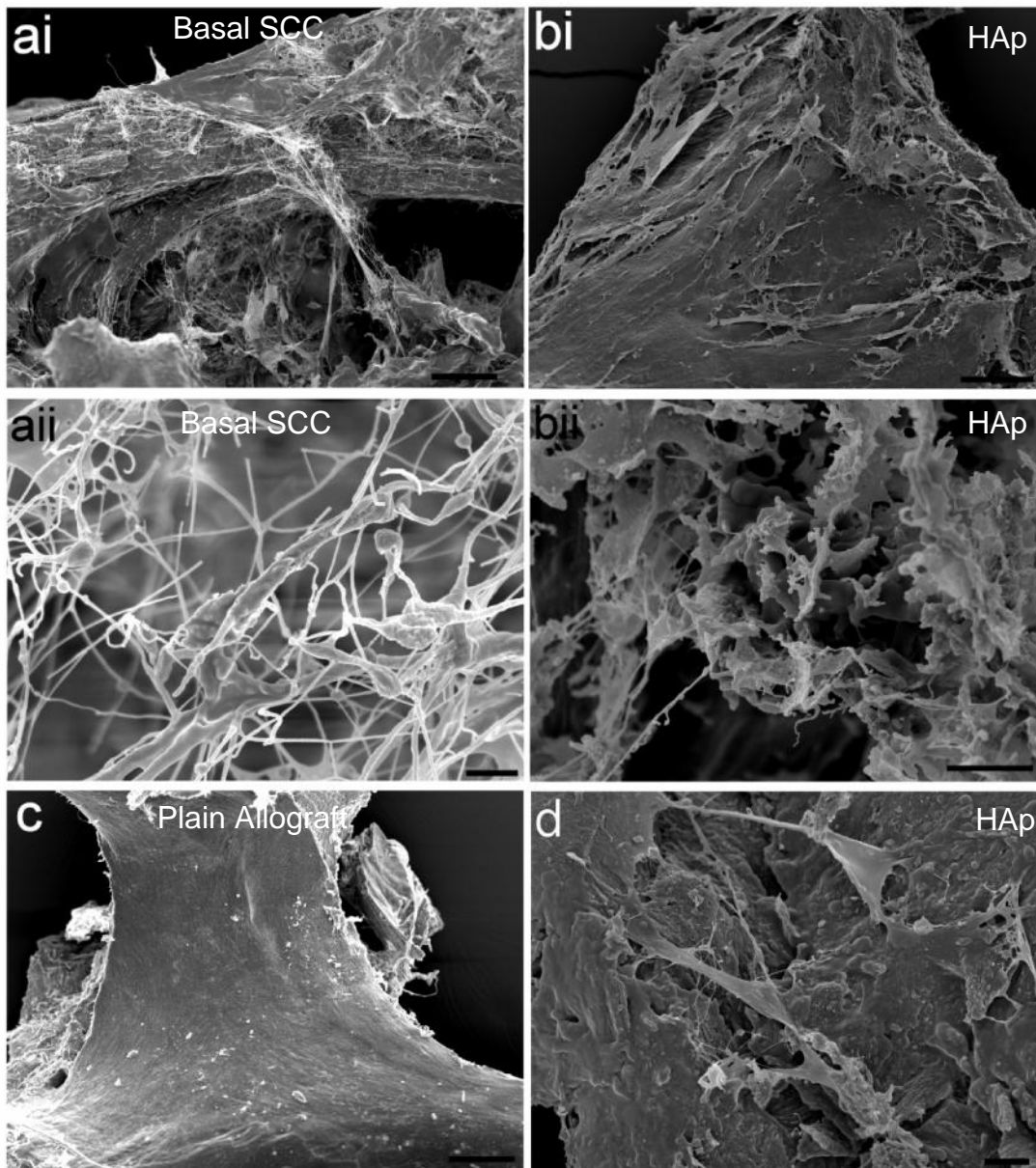
Fig 6:9 SEM micrographs of IBG samples (a) Basal control SSC showing cells and matrix covering allograft. (b) HA nanoparticle group with cells and matrix coating the allograft (c) plain allograft showing no covering to bare trabeculae.. Scale bar 100 µm (aii) and (bii Higher magnification of Basal SSC control and HAp samples respectively showing matrix forming spherulite like particles thought to be an early marker for

mineralisation. Scale bar 4 µm, (d) Further SEM image of HAp-SSC sample showing adherent cells and matrix bridging across to separate allograft fragments. Scale 10 µm.

The newly formed matrix on the allograft surface showed signs of mineralisation including the formation of spherulite-like particles, thought to be associated with the initial stages of mineralisation (Poulsson, Mitchell et al. 2009). Although these were observed in both HA and basal samples, the apparent mineralisation is more extensive in the former (Figure 6:9 aii, bii).

6:8:5 FTIR spectroscopy

Comparison of HA-SSCs with the basal SSC control, suggests a higher mineral content in the nanoparticle-treated samples. The mineral to matrix ratio for HA-treated samples was found to be comparable with that obtained from the original allograft (Figure 14). This difference in composition may be significant in influencing the mechanical properties of the impacted sample. Carbonates to mineral ratios were also found to be the same for the allograft and HA-treated IBG samples.

	Matrix	Mineral
Basal	1	2.40
HAp	1	5.25
Allograft	1	5.25
	Mineral	Carbonate
Basal	1	0.022
HAp	1	0.023
Allograft	1	0.023

Table 6:2 Relative composition of cultured IBG samples from FTIR analysis.

6:9 Discussion

The results from this array of investigations are encouraging for the use of HA nanoparticles to enhance SSC differentiation down an osteogenic lineage. Alkaline phosphatise stain and assays have both shown significant increase in activity levels. Electron microscopy images have shown signs of increased mineralisation within the HAp group compared to the control and this would support the results of mechanical testing in which the HAp group had a significantly higher shear strength and interparticulate cohesion. As the SSC divide and cover the surface of the allograft chips, and the extracellular matrix deposited starts to mineralise, so the bond between the particles increases resisting the shear forces applied.

FITR Spectroscopy has also shown increased mineralisation with a higher mineral to matrix ratio compared to the basal controls. DNA concentration was not significantly different from the controls suggesting there was no enhancement in SSC attachement or rate of proliferation, which was as expected.

Previous work (Babister et al 2009; Gonzalez-McQuire et al 2007) has focused on the 2D and 3D culture of SSC and HAp with significant improvement in the osteogenic differentiation. To my knowledge this is the first study to show that these observed effects can be extrapolated to use with allograft in IBG and have a pronounced effect on the invivo mechanical strength.

Although the HA nanoparticles used in this study are not yet approved for clinical use, the transfer of these techniques into theatre should be feasible although access to a centrifuge and a tissue culture incubator would be required in the vicinity.

One concern that had been raised during presentation of these results was the safety of having IGB containing the HAp cultured nanoparticles in the vicinity of a highly polished new hip replacement as a potential source for third body wear and or immune response. Although there is likely to be some excess particles during the initial mixing phase and culture, the samples then undergo centrifuge and repeated washing which is likely to reduce the 'free floating' debris significantly. It is also worth noting that the nanoparticles do not appear to stay on the surface of the cells, but rather are taken up and incorporated by the SSC (see figure 6:5).

For transfer to clinical use, further work would also be required on analysing any distant effect of the nanoparticles as the effects on other cell types and transfer to distant organs is not fully understood. Due to the ability to attach various agents to the HAp, their use as vectors for delivering therapies has also been studied and one study looking at the effect of HAp on pulmonary surfactant showed significant changes to the lining of the respiratory tract after exposure to HAp (Qihui Fan et al 2011). Whilst the HAp used for this experiment is in solution and not necessarily of similar form, it does highlight the need for further studies before clinical application is considered.

The mechanism by which the HAp particles act to induce osteogenesis is still not fully understood. Various techniques using florescent labelled probes attached to the HAp particles to try and demonstrate the reaction pathway in living cells have been tried but as yet, unsuccessful (Tanaka et al 2001; Zumbuehl et al 2004; Tatton et al 1997).

There is considerable development of a huge variety of Hydroxyapatite structures and HAp nanoparticle compounds at present with a large number of patents being filed for the differing structures (Yurong c et al 2008). Ostim is a commercially available compound made up of crystalline HAp that comes in paste form and has been used to good effect in bone regeneration for periodontal defects (Heinz B et al 2009).

6:10 Summary

HA nanoparticles pose an exciting potential for regenerative treatment for bony defects. Hydroxyapatite already has a good reputation for the coating of implants so transferring it's potential osteogenic benefits in to clinical practice should not be too much of a psychological step change for the orthopaedic surgeon. The main disadvantage with the current technique is the requirement for tissue culture and incubation which could all be potential sources of contamination along with the logistical issues.

6:11 Conclusion

Null Hypothesis

- Coating of milled allograft with SSC cultured with HAp nanoparticles will not improve the osteogenic differentiation of the cells on the construct

    This hypothesis has been shown to be false

- Coating of milled allograft with SSC cultured with Hap nanoparticles will not enhance the mechanical properties of the construct
    a. This hypothesis has also been shown to be false

Chapter 7

In Vivo Study on the biological effects of Type 1 Collagen and Hydroxyapatite on SSC IBG construct

7:1 Introduction

The in vitro studies have demonstrated the potential for both Type 1 Collagen and HA nanoparticles to enhance the osteogenic differentiation of SSC progenitors. In the setting of Impaction bone grafting this is highly desirable. To assess the potential for these techniques to be transferred into the clinical setting it was deemed appropriate to assess the in vivo response using an established mouse model (Bolland et al. 2008). The control of osteogenic differentiation and new bone formation is a complex process involving many synergistic processes under the control of various growth factors and hormones as described earlier. It is impossible to replicate these effects fully in the in vitro setting thus the need to transfer to a live animal. For the purpose of this experiment a mouse model was used. There are many advantages to using the mouse model initially, including cost and time issues, but the model is not without its disadvantages. The samples were contained within modified electron microscopy pots to maintain containment of the IBG construct, and whilst they will be subjected to the various hormonal stimuli from the host animal, there is no mechanical strain or stimulation of the tissue. Ultimately to replicate the clinical setting for IBG, the samples need to be under cyclical loading in a contained bony cavity with bone to IBG and IBG to cement interface. This is not feasible for a mouse model and is best reproduced using a sheep hemiarthroplasy model, but due to various factors this was not possible during my period of study, but large animal studies are now underway.

7:2 Aim

To analyse whether the observed in vitro benefits of Collagen coated allograft and HAp cultured SSC have the same effect on the osteogenic differentiation under physiological conditions in an in vivo mouse model.

7:3 Null Hypothesis

HAp cultured SSC and Collagen coated allograft has no effect on the osteogenic differentiation of SSC when cultured in an in vivo subcutaneous mouse model

7:4 Materials and Methods

Separate allograft samples were prepared, seeded and impacted as previously described, but using scaled down chambers appropriately sized for the mouse dorsum. Electron microscopy pots were modified to form perforated capsules containing $1cm^3$ of graft using a scaled-down impactor to replicate the femoral IBG process. Allograft samples were seeded with SSCs, impacted and cultured *in vitro* overnight before implantation.

Male MF-1 nu/nu immunodeficient mice were purchased from Harlan, Loughborough, UK and acclimatized for a minimum of 1 week prior to experimentation. All procedures were performed after prior ethical approval in accordance with regulations specified by the Animals (Scientific Procedures) Act 1986.

A total of 16 mice were used for this experiment with 4 groups:

    (i)   Plain Allograft

    (ii)   Plain Allograft and Basal SSC

    (iii)   Collagen Coated Allograft and Basal SSC

    (iv)   Plain Allograft and HAp cultured SSC

8 capsules were used for each group in total, with 5 for biochemical analysis, and 3 for histological analysis.

The animals were anaesthetised with fentanyl-fluanisone (Hypnorm) (Janssen-Cilag Ltd, High Wycombe, UK) and midazolam (Hypnovel) (Roche Ltd, Welwyn Garden City, UK) in sterile water at a ratio of 1:1 and a dose of

10ml kg^{-1} intraperitoneally. The capsules were implanted subcutaneously on the rear flank of the animal.
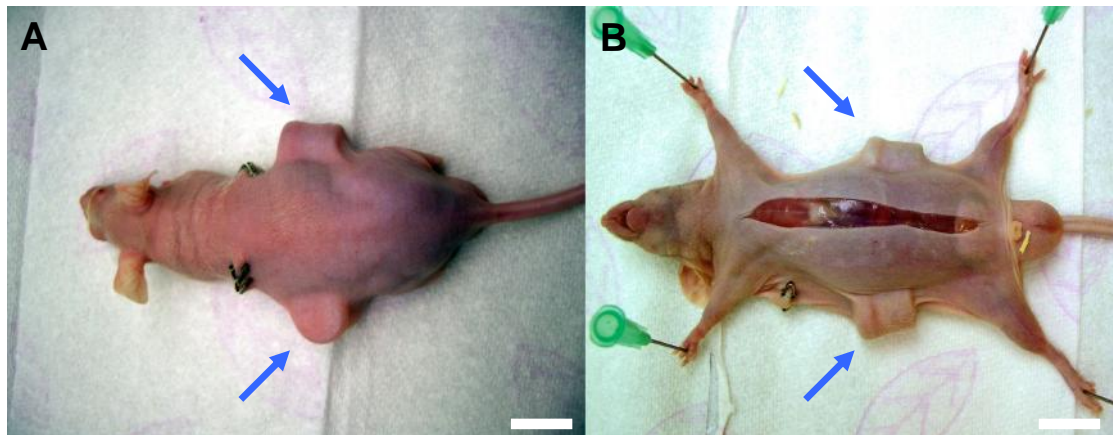
Fig 7:1 A: Mouse with 2 Capsules implanted subcutaneously in both flanks. B: After 28 Days the animals were euthanized and a midline sternotomy and laparotomy performed. Scale bar 10mm

After 28 days the animals were anaesthetised and a midline sternotomy was performed. The left ventricle was cannulated and perfused with heparinised normal saline until clear blanching of the liver occurred (Figure 7:2B). A pre-prepared solution of Microfil (consisting of a lead chromate-containing radio-opaque polymer, Microfil MV-120 blue and MV-diluent solutions (1:1), MV-curing agent (10% of total volume)) was infused at a constant rate and pressure until the dye was visible in the ear, tail and mesenteric veins (figure 7:3A&B), indicating complete perfusion of the peripheral circulation. The samples were then harvested (figure 7:4), fixed in paraformaldehyde 4%, scanned in using an Xtek Benchtop 160Xi scanner (Xtek Systems Ltd, Tring, UK) equipped with a Hamamatsu C7943 x-ray flat panel sensor (Hamamatsu Photonics, Welwyn Garden City, UK) followed by decalcification for histological analysis as described .
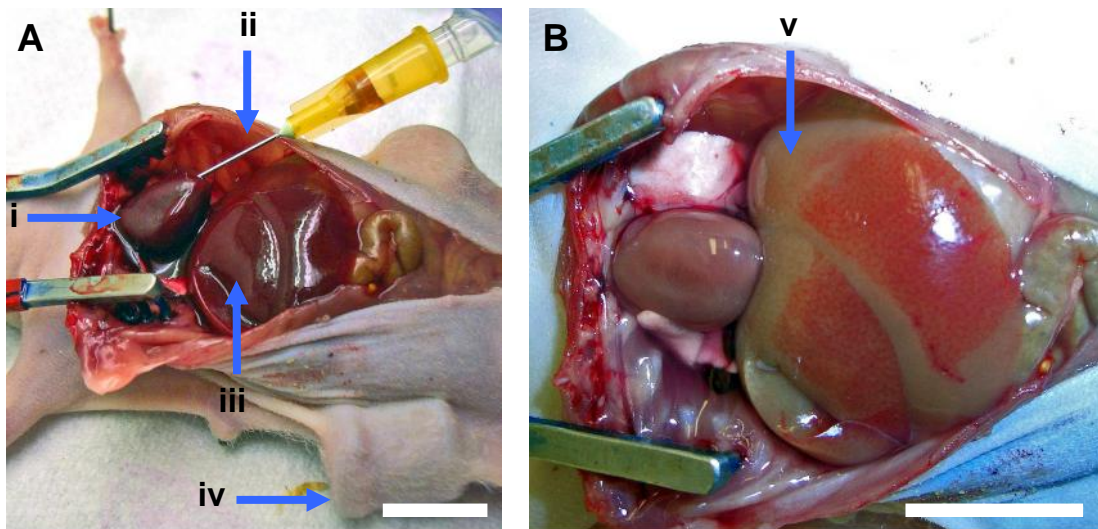
Fig: 7:2

A: Mouse post laparotomy and midline sternotomy (i) showing heart and (iii) liver. (ii) Injection of heparinised saline with 22 Gauge needle into left ventricle. (iv) Subcutaneous capsule containing IBG and SSC.

B: Close up of Heart and Liver following initial flushing of vascular bed with heparinised saline showing blanching of liver from the blood filled state seen in (iii). Scale Bar 10mm.
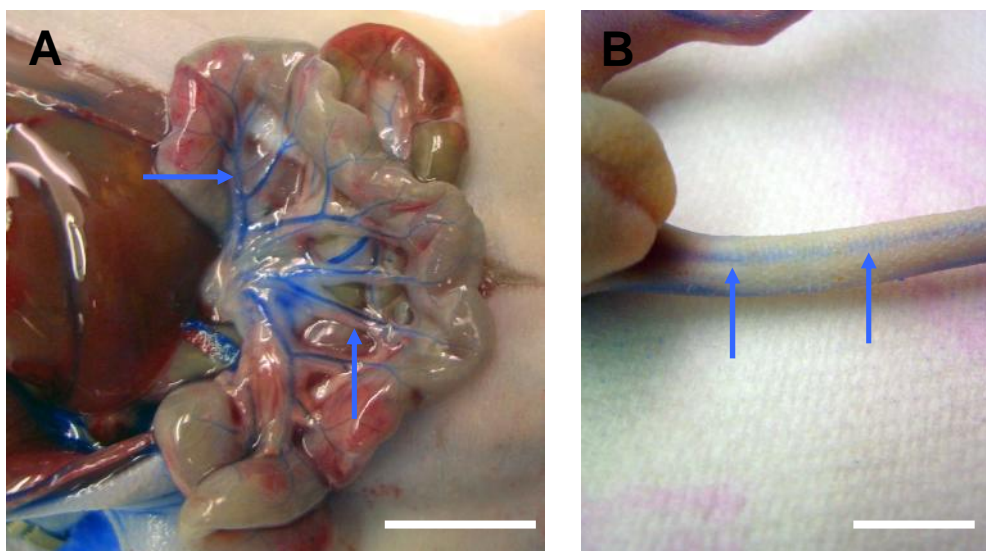
Fig 7:3

A: Mouse intestine post infusion of microfil demonstrating complete filling of mesenteric vessels

B: Mouse tail showing vessel filling with microfilm. Scale 10mm

7:5 Results

7:5:1 New Blood Vessel Formation

Surgical dissection of the plastic capsules revealed a tissue capsule containing multiple fine vessels surrounding the sample and penetrating through into the graft bed. Although this was the case for the majority of the capsules, some where observed to be relatively avascular with little or no signs of new vessel formation or tissue reaction.

The Macroscopic appearance of in vivo capsules post culture are seen below.
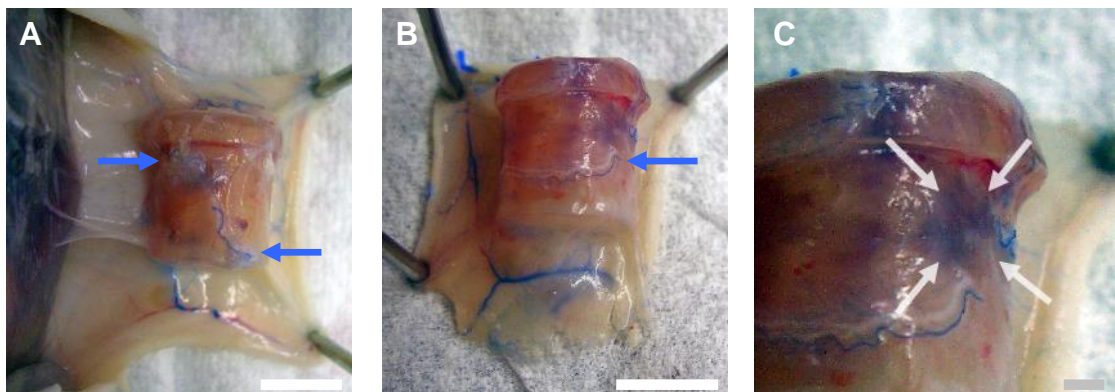
Fig 7:4 Photos showing IBG pot harvest.
A: Capsule was first dissected off the mouse flank with the subcutaneous tissues and skin. B: Sample separated from mouse. Blue arrows showing microfilm within new vessel formation surrounding capsule.
C: Close up photo showing blue blanching inside capsule signifying vessel penetration into graft bed highlighted by white arrows. Scale bar: A&B 5mm C 1mm

The thickened capsule surrounding the capsules was maintained to preserve the fine blood vessels. The capsules then underwent decalcification as previously described before sectioning for histology.

7:5:2 Histology of Capsules
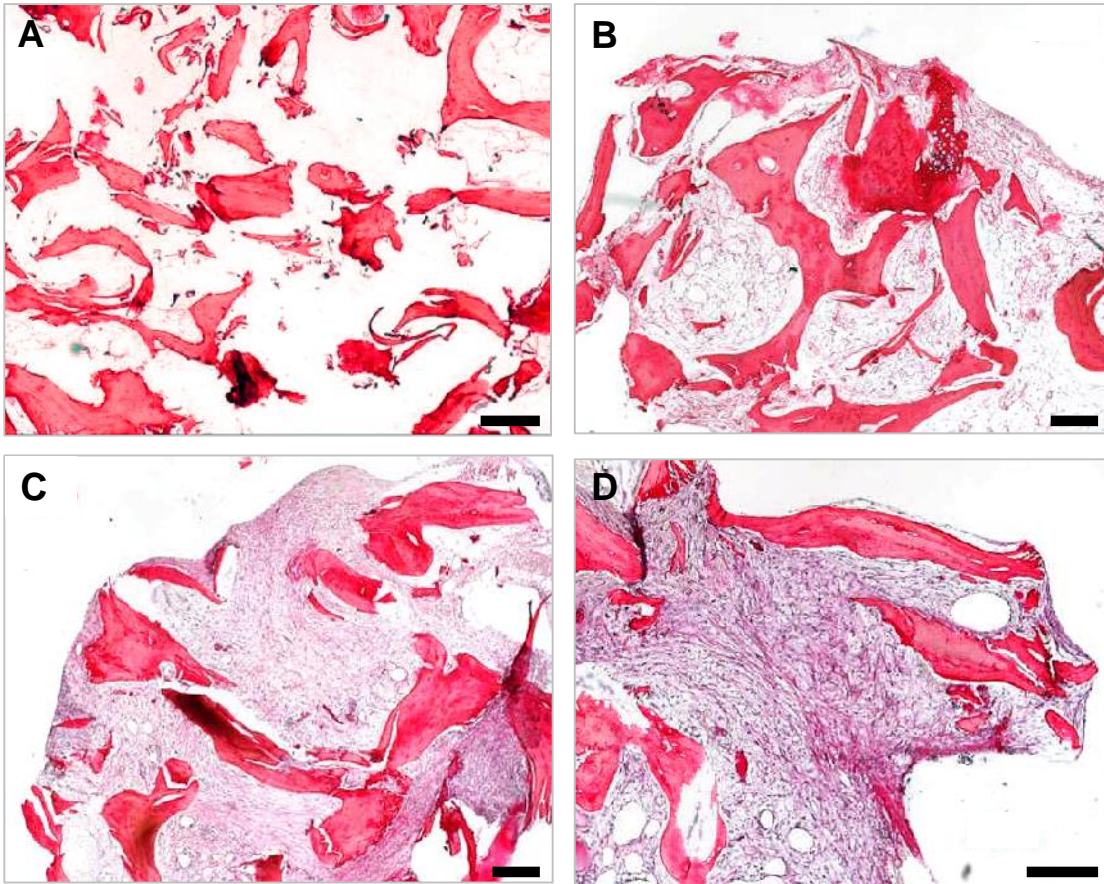
Fig 7:5 Microscopy views of sectioned samples following A&S staining

A: Plain Allograft sample. B: Plain Allograft and SSC. C: Collagen coated Allograft and SSC. D: Allograft and HAp cultured SSC. Scale bars 500µm
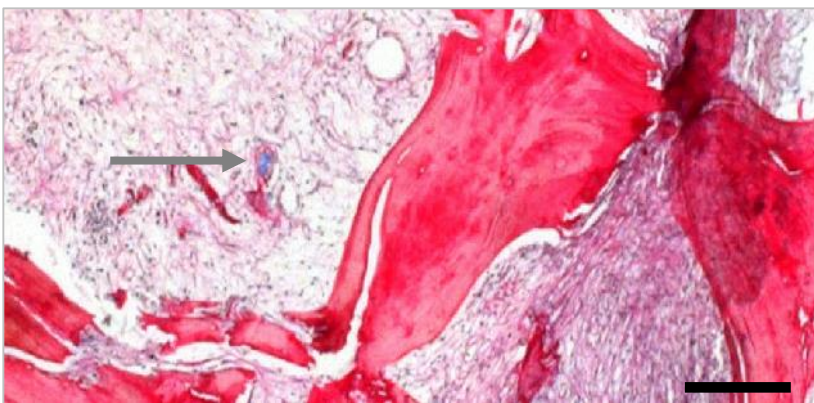
Fig 7:6 Higher Magnification of Collagen coated sample showing small blue microfilm of a new vessel in growth within new cellular matrix.

Immuno histochemical staining for Col 1 was performed on all samples looking for evidence of increased osteogenic differentiation. Unfortunately none of the antibody stains would work and on discussion with my supervisors it was thought the prolonged decalcification period may have destroyed the markers although the Tris / EDTA used had not caused any problems with previous experiments and immunohistochemical staining.

7:5:3 Biochemistry of subcutaneous IBG samples

Pico Green DNA assay

Five capsules from each group were immediately placed in basal culture media on harvesting from the mice and transferred back to the lab for biochemical assays to assess cell proliferation and osteogenic differentiation with Alkaline Phosphatase activity.
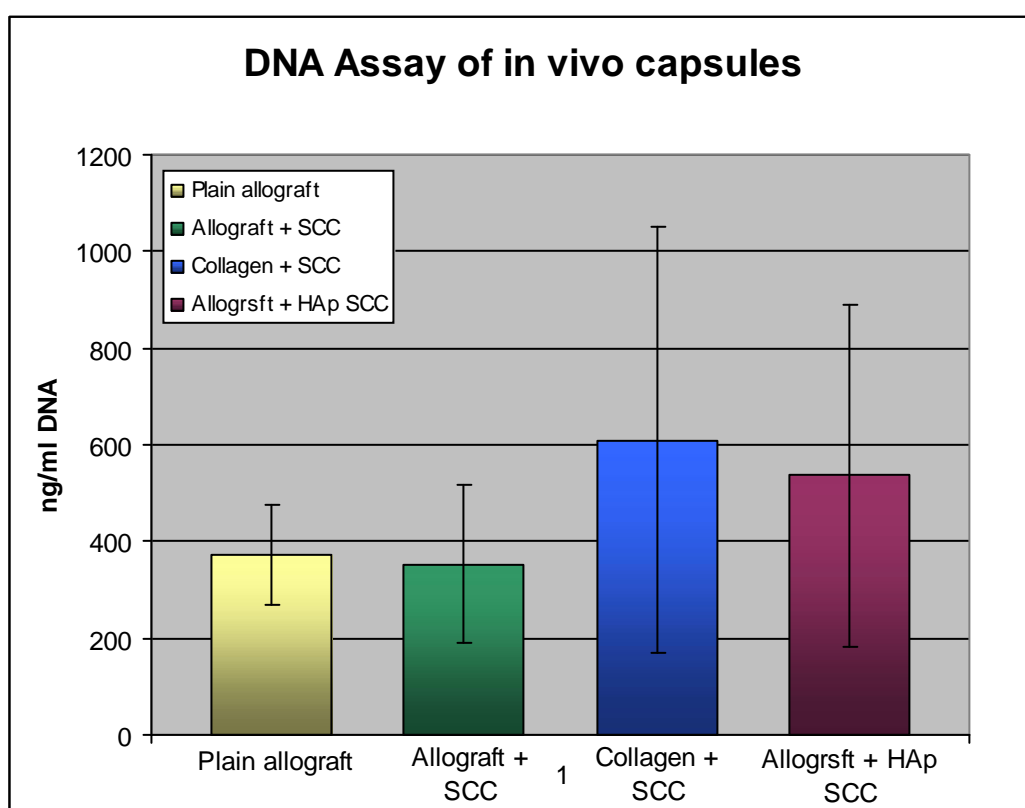
Fig 7:7 Pico green assay of allograft and allograft + cells as controls and the experimental groups of Collagen and HA nanoparticles. N=5. P>0.05

The above graph demonstrates no significant difference in the DNA content of the 4 groups with fairly marked variability within the groups as signified by the large error bars (+/-1 SD)

Alkaline phosphatase assays were also carried out for the samples. Unfortunately the values were negligible across all samples, with any positive amount within the usual baseline readings for plain allograft. The results have therefore not been included for formal analysis

7:6 Discussion

Alcian blue and Sirrus red staining reveals minimal cellular material in the plain allograft explanted group and progressively more staining in the allograft plus cells, type I collagen and HAp groups. Whether this represents host vascular cells or culture-expanded allogeneic SSCs is unclear, but either of these attributes would be beneficial for the IBG construct in a clinical scenario.

Furthermore, by Microfil perfusion of the circulatory system of the mouse, and post-mortem analysis of the harvested subcutaneous capsule, new vessel formation is demonstrated, a critical step in the tissue engineering pathway.

The data obtained from this experiment were not as planned with the key biochemistry and immunohistochemisrty failing to work. One of the main factors for the Ab staining was thought to be secondary to the prolonged time it took to get the samples to decalcify enough to allow sectioning, despite repeated changing of the solutions. In hindsight keeping the pseudocapsule in place to preserve the fine vessel matrix growing into the capsule may have prevented de mineralisation, but the initial concern was to avoid any damage to the fine de novo vessels.

The biochemical analysis was hampered by very variable results, and this may well be due to the findings noted at time of surgical dissection. A few of the capsules appeared to have little in the way of a reaction surrounding them with little or no blood vessels, occurring in all experimental groups. In effect there was insufficient delivery of oxygen and nutrients to the capsule in the initial phase and we postulate that the SSC failed to proliferate. It may also be the case that due to the high concentration of the SSC, the metabolic demands of some of the samples may have outstripped local supply causing subsequent cell death.

Despite the results obtained for this experiment, the murine model used has worked well in the past. Bolland et al has demonstrated that SSC seeded allograft implanted subcutaneously does proliferate, and also stimulate new blood vessel formation with significantly more and larger vessels entering the sample compared to plain allograft. His study involved the use of SSC cultured in osteogenic media to increase osteogenic differentiation. This is not readily transferable to the clinical setting due to the side effects of the steroids used. The model has also been used with synthetic scaffolds and SSC in combination (Bolland et al 2008; Aarvold et al 2010) both demonstrating viable SSC replication and osteogenic differentiation. The aim of this study was to demonstrate that Collagen coating of the allograft, and HAp seeding of the SSC can enhance the ongoing osteogenic differentiation of the SSC to improve new bone formation.

An in vivo model is likely to increase the variability of results compared to a controlled in vitro setting. Unfortunately it is difficult to draw any strong conclusions from the results of this experiment, with the reasons for failure being multifactorial. Ideally the next phase of in vivo analysis will more closely replicate the clinical setting of IBG with sufficient blood supply to support the SSC / IBG construct.

7:7 Conclusion

Null Hypothesis

HAp cultured SSC and Collagen coated allograft has no effect on the osteogenic differentiation of SSC when cultured in an in vivo subcutaneous mouse model

Due to the problems outlined above we have been unable to refute the null hypothesis for this experiment.

Section III

A Novel Impaction Bone Grafting Technique

Chapter 8

From Roadside to Revision Hip Surgery, the Role of Vibration Impaction in the Acetabulum

Chapter 8

From Roadside to Revision Hip Surgery, the Role of Vibration Impaction in the Acetabulum

8:1 Introduction

Impaction bone grafting of fresh frozen allograft remains the gold standard for replacing lost bone stock found during revision hip surgery. Survivorship for acetabular and femoral components at 20 and 10 years respectively is encouraging (87% and 99% although such results have not always been repeated outside specialist centres (Eldridge et al. 1997; van Haaren et al. 2007). It is however a technically demanding procedure with a significant learning curve and as such its use outside of centres of excellence is not widespread. Loosening of a total hip replacement can lead to a significant loss of bone stock with only thin cortical walls left in place. The need to provide primary stable fixation of the revision implant is critical for the long term success and secondary bony in growth. This leads to a situation where sufficient compaction of the allograft construction is required, often requiring high forces, imparted into a fragile host bone stock. There is therefore a thin line between risk of fracture to the native bone and insufficient compaction of the IBG bed.

The compaction of aggregates is a familiar process in civil engineering to provide amongst other things, roads and foundations. The mechanical properties and ways to optimise the aggregate base have been extensively studied (Smith GN. 2006), and these can be potentially transferred into IBG. The aggregate should be adequately compacted and built up in layers using a suitable compaction force to enable uniform compaction throughout a thick layer of aggregate. The aggregate should be composed of different particle sizes or 'well graded' to ensure that there are large particles to form significant structural integrity, and smaller particles to fill the voids in between (Brewster et al 1999, Dunlop et al 2003). The aggregate also needs to be sufficiently

contained, as otherwise sequential impaction will lead to continued escape from the breach, which in clinical terms could mean an un noticed filling up of graft into the pelvic cavity. Sufficient force also needs to be applied, and it is this parameter that is often difficult to determine clinically.

Vibration impaction has been used in civil engineering for a long time (Smith G.N 2006) for the successful compaction of aggregates. If vibration is used within a confined space, certain factors have to also be considered. Inevitably there is a fluid element to the aggregate (particularly in the setting of IBG) and if vibration is applied in this situation a fluid pressure can build up. If there is significant build up of fluid pressure during the vibration impaction then this can start to disrupt the compaction of the solid aggregates and a process of liquefaction occurs. This process can often occur during earthquakes when the aggregate base below structures changes into this liquid state and the structural supportive integrity is lost and the building appears to sink. Drainage during vibration impaction is therefore a key factor.

Vibration impactions use as a potential surgical technique in the femur has been studied and shown to offer significant advantages both in the compaction of the graft and reduction in the forces required to achieve this (Bolland et al, 2008). Acetabular impaction differs from the femur in that the defect and anatomy are more open and not a narrow blind ended tube, and the graft bed can often need to be significantly thicker with large cavitatory defect caused. The process of standard impaction also less controlled, for a femur a drop weight is attached to the tamp to try and standardise the forces, but this is not the case for Acetabular impaction due to the multiplanar direction of impaction required.

8:2 Aim

The aim of this study is therefore to see if the benefits of vibration impaction can be applied to acetabular IBG to reduce impaction forces and improve graft compaction and cup stability

8:3 Null Hypothesis

The use of vibration impaction in acetabular IBG does not reduce the forces imparted to the acetabulum.

The use of vibration impaction in acetabular IBG cause insufficient graft compaction and as such risks cup subsidence

8:4 Materials and Methods

8:4:1 Pelvic Model

For the purposes of this study and to obtain reproducible data, sawbone biomechanical hemi pelvis models were used (Sawbone 3rd Generation).
The acetabular defects encountered during revision surgery have been classified according to there morphology by Praposky. (Table 8:1)

Defect	Rim	Walls	Columns	Bone Bed
Type 1	Intact	Intact	Intact	>50%; cancellous
Type 2	Distorted	Distorted	Intact	<50%; cancellous A= Superomedial B= Superolateral C= Isolated Medial
Type 3	Missing	Severely compromised	Non-supportive	Membranous/ sclerotic

Table 8:1 : Paprosky classification of acetabular defects and treatments guidance. (Paprosky, Perona et al.)

These defects can be either contained or uncontained, but in order to proceed with IBG the uncontained defect needs to be reconstructed for example with a mesh. To avoid unnecessary variables a contained cavitatory defect (Praprosky type 2a) was used for this experiment.

Using a custom made jig (Southampton Bioengineering Dept) that allowed multiplanar positioning of the hemipelvis, the standard anatomical acetabulum was reamed using a set sequence of reamers to form a supero medial cavitatory defect similar to those found in clinical practice. The volume of the cavity was 75cm^3 with each hemipelvis prepared in identical fashion.
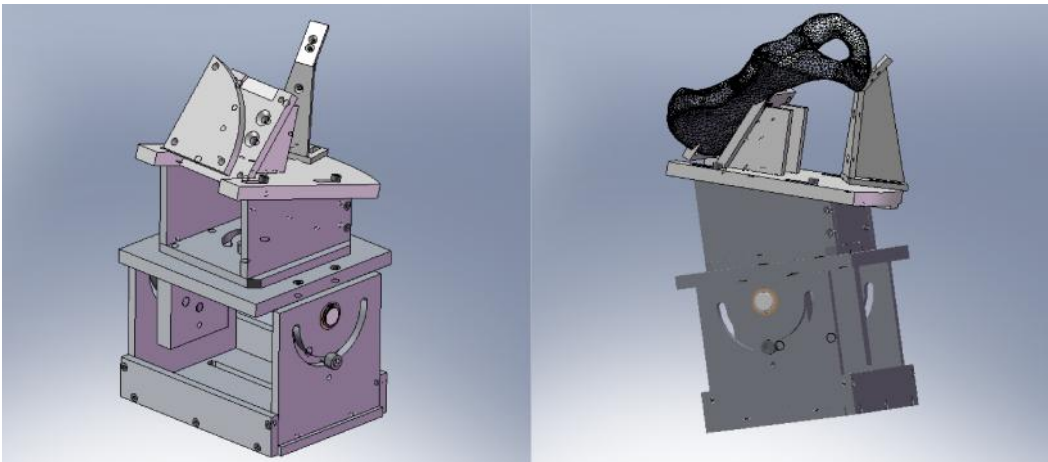
Figure 8:1 : Schematics of custom jig to allow multiplanar positioning of hemipelvis model
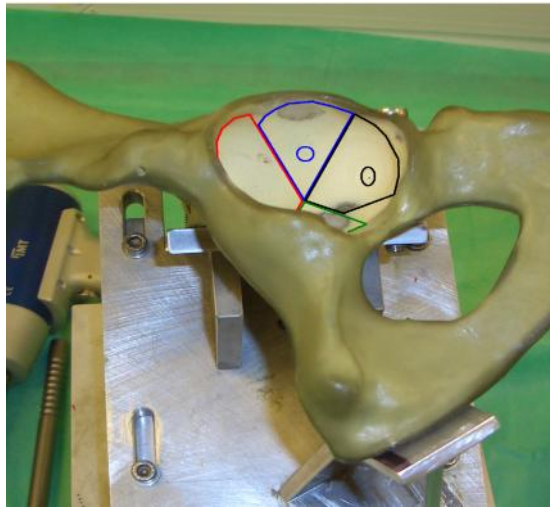
Figure 8:2: Hemipelvis on Jig with reamed out cavity. Coloured sectors in cup represent the 4 different reamers used in sequence to achieve a large, reproducible cavitatory defect.

Stryker Xchange tamps were used for the impaction process. These were modified from standard with multiple drainage holes to allow any fluid to collect within the tamp and prevent the process of liquefaction occurring during vibration impaction (Smith 1990) Fig 8:3.

The tamps were connected to a device known as the 'Woodpecker' (Minnesota Bramstedt Surgical Inc, St. Paul, MN, USA). It is a pneumatic hammer designed primarily for use in hip replacement surgery for broaching of the femoral canal. Initial work by Bolland et al established the optimal pressure for the puposes of impaction grafting as standard broaching forces were too high. The pressure used for this experiment via a regulator waspressure regulator was 2.4 bar with the control on the body of the Woodpecker set in the maximum (+) position (Fig 8:3 B). The frequency of the vibrations delivered by the Woodpecker device were at a rate of 70Hz. The coupling between the tamp and Woodpecker was a simple sleeve that ensured the force of vibration was transferred towards the graft bed, but on the 'backward' oscillation of the device the tamp was not actively retracted by the coupling so as not to disrupt the graft bed.
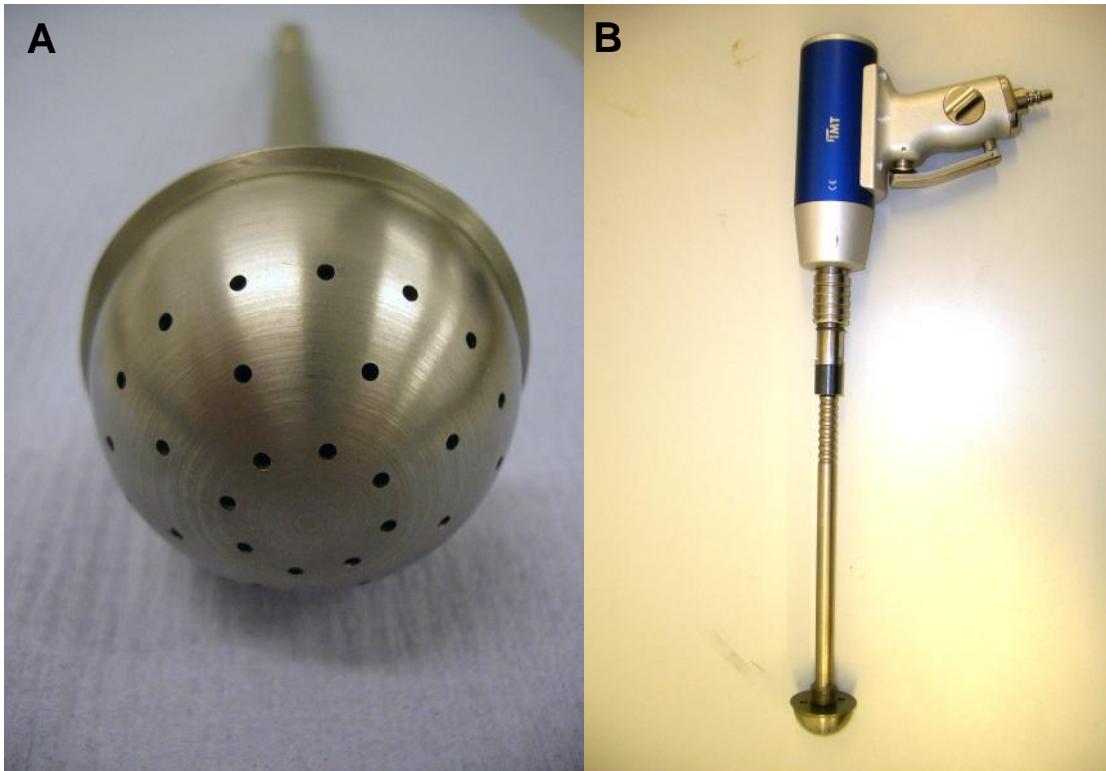
Figure 8:3: A: Stryker Xchange tamp modified with drainage holes. B: Tamp attached to Woodpecker device for generation of vibrations. Coupling between the two was not fixed to prevent retraction of tamp on 'backstroke' of vibration impaction and subsequent disruption to graft base.

8:4:2 Defining Adequate Compaction

Defining adequate compaction of the allograft base intra operatively is a difficult skill. In Femoral IBG this is readily defined as no further subsidence of the tamp after 10 successive blows. In the acetabulum however this is not as straightforward as the change in depth of the tamp is minimal. In clinical practice, the graft base is palpated with the surgeon's finger and the tactile feedback and experience of the surgeon is used to determine when it is sufficiently solid to progress on to the next layer. Experimentally this is hard to quantatively define, it was therefore necessary to come up with a quantifiable, and reproducible method of determining graft compaction 'intraoperatively'.

A device known as a penetrometer is used in engineering to determine the compaction of aggregates such as soil. I therefore proposed that this could be transferred to determine allograft compaction. A pen type penetrometer was modified by reducing the tip diameter to 3.2mm diameter to allow penetration into the graft bed without complete disruption. See Figs 8:4 & 8:6. In order to validate the penetrometer an initial experimental run was set up to correlate the penetrometer readings to an experienced surgeon's determination of sufficient compaction.
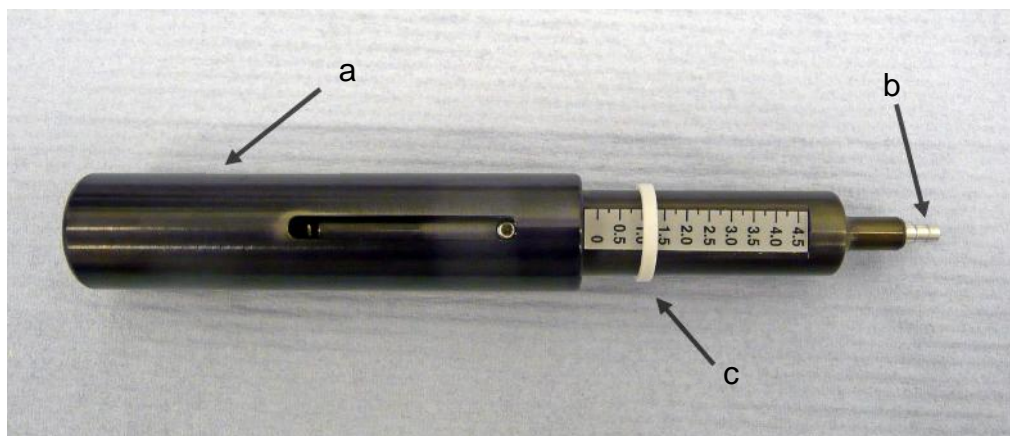
Fig 8:4 Modified penetrometer. a: spring loaded outer sleeve that is pushed down on until tip of penetrometer – b penetrates the graft bed up to the second indent. c: mobile ring from which maximal reading is taken.

8:4:3 Allograft

Allograft used for this experiment was prepared in standard fashion as previously described in 2:1. The allograft was defrosted and brought up to room temperature and the experiment performed in a temperature and humidity controlled laboratory. The volume and weight of allograft was standardised for all experimental runs

8:4:5 Strain Gauge

For the Experiment the hemi pelvis was mounted in the jig held with an epoxy resin plate contoured to the sacroiliac joint and held against a small rubber coated metal plate at the pubis to represent the normal anatomical attachments of the pelvis.

A single rosette strain gauge was fitted to the medial wall of the inner pelvis to measure the stress imparted to the wall of the pelvis (Fig 8:5). The gauge was secured with cyanoacrylite glue (locktite 'Superglue') and covered in silicon sealent to prevent damage from any water ingress. The position of the gauge was measured and identical for each experiment.
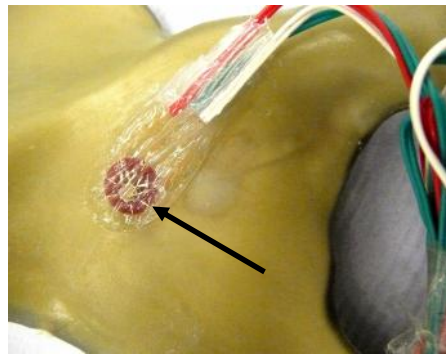
Fig 8:5 Rosette strain gauge attached to the inner aspect of the medial acetabular wall and covered in silicone gel to prevent fluid ingress

8:4:6 Study Design

To simplify the experimental process the IBG was divided into 6 steps for each experimental run. A number of preliminary experimental runs were performed to confirm the most appropriate amount of graft to be added at each step and the impactor sequence to give a reproducible and standardised experimental run for each group. The final sequence was as follows:

        Step 1 - 50ml Graft. 46mm Impactor tamp

        Step 2 - 25ml Graft. 46mm Impactor tamp

        Step 3 - 25ml Graft. 46mm Impactor tamp

        Step 4 – No Graft. 50mm Impactor tamp

        Step 5 – 25ml Graft. 46mm Impactor tamp

        Step 6 – No Graft. 50mm Impactor tamp

To mimic the clinical phenomenon of blood pooling at the base of the IBG bed during impaction 3ml of Fetal Calf Serum (Sigma Aldridge) was added before steps 2 + 3. This was felt to give a similar viscocity to blood.

Strain gauge measurements were recorded throughout the six impaction steps using a computer equipped with a data acquisition card and signal conditioning unit (DAQCard-AI-16XE-50 and SC-2345, National Instruments Ltd, UK). Data was sampled at a frequency of 10 kHz to ensure capture of the high frequency components of the load and strain signals produced by the impaction. The data was recorded using National Instruments VI Logging software.

Penetrometer readings were taken from five set zones within the acetabular IBG bed to confirm sufficient compaction before proceeding on to further addition of graft and impaction.
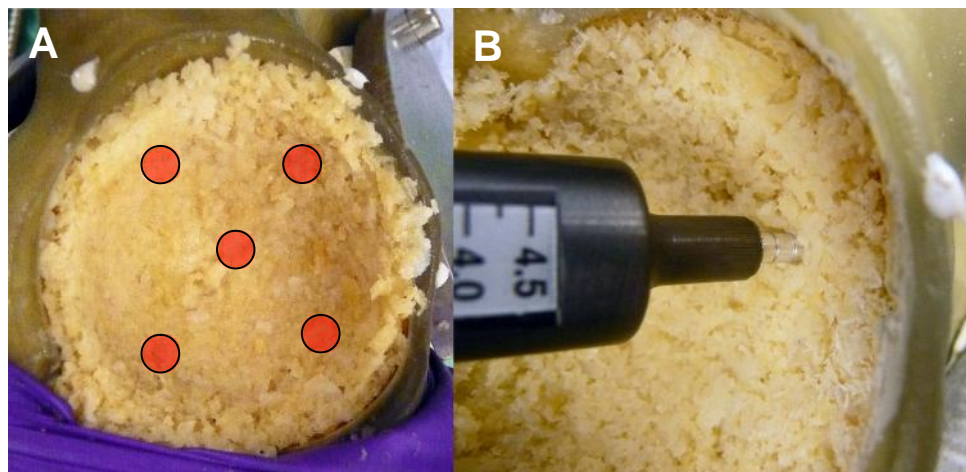
Fig 8:6 A: IBG graft bed with the 5 zones of penetrometer readings signified by the red dots. B: Example of penetrometer reading with fine tip pressed into the IBG bed until the edge was level with the second indent. Measurements were then taken from the scale on the side

8:4:7 Cementation

Once the final graft bed had been established, a single mix of bone cement (Smartset CMW, DePuy CMW Ltd., Blackpool, UK),was prepared in a vacuum mixing system (Cemvac, DePuy CMW Ltd, Blachpool, UK) as per standard techniques. The cement was introduced into the acetabular cavity at 3 minutes and pressurised in standard fashion with a size 56 pressuriser (Stryker, Howmedica) for 1 minute

8:4:7 Cup Implantation

Size 48 / 28 Flanged Exeter Contemporary cups were used for all experimental runs. The flange was cut to < 2mm rim to prevent any direct contact with the acetabular rim. Although in normal clinical practice the flange is contoured such that it rests on the acetabular rim it was felt that this would potentially mask any small differences in the ability of the graft bed to provide structural support during the cyclical loading. Thus as the experimental aim was to test and differences in the graft bed and not the cup / cement / bed construct as a whole, this was standardised for all.

To ensure standardisation of the cup positioning, the classical position of the cup in 45Deg Adduction and 30 Deg Flexion (Exeter Hip techniques) was used with the hemipelvis orientated on the jig such that this cup position corresponded to vertical alignment of the custom cup holder. The accuracy of positioning was within 0.5 Degrees

To standardise the depth of the cup implantation a custom cup holder was manufactured. This composed of a metal rod and plate with pins to locate into the cup, and a mobile acrylic ring that could slide up and down the shaft. The acrylic ring when placed on the 'bony' prominences of the acetabulum ensured identical alignment of all cup implantations, with a marker on the shaft to standardise the depth. See Fig 8:5
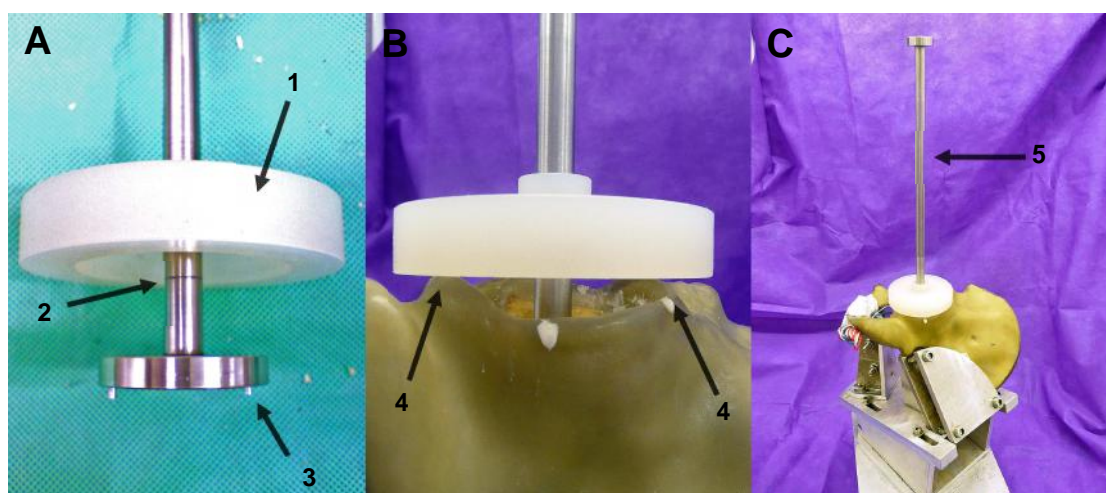
Fig 8:5 Cup implanter. A: 1: Mobile Acrylic ring. 2: Etch mark on shaft to mark depth of cup implantation. 3: Small prongs to engage with cup. B: 4: Mobile acrylic ring pushed down onto bony prominences to fix angle of implantation. C: Whole jig arrangement with cup introducer in situ 5: Shaft kept vertical to maintain constant / reproducible cup orientation

8:4:8 Mechanical Testing

Industry standard testing parameters have been described for the testing of Femoral components and were used in previous studies on the effects of vibration impaction in the Femur (Bolland). Although no such standards exist for acetabular testing the cyclical loading pattern as described by (Morlock *and Westphal et al., 2006)* was deemed appropriate and used initially (Table 8:2).

Sequence	Number of Cycles	Minimum Load (N)	Maximum Load (N)	Mean Load (N)	Load Amplitude (N)
1	20	50	200	125	75
2	980	50	800	425	375
3	1000	50	1200	625	575
4	1000	50	1600	825	775
5	47000	50	2100	1075	1025

Table 8:2 Cyclical loading protocol as per Morlock and Westphal et al.

The maximal loading of the acetabulum occurs during heel strike. The angle of force during was calculated in relation to the hemipelvis model and the construct re orientated on the jig such that this was in the vertical plane. An Instron 8878 servo-hydraulic materials testing machine (Instron Ltd, High Wycombe, UK) was used to load the test rig cyclically. This was achieved via attacement of a standard 28mm V40 Exeter Stainless Steel Femoral head to the Instrom which was then lowered into the cup and the whole construct fixed in place. Axial displacement / Subsidence of the cup was measured directly from the Instron transducer.
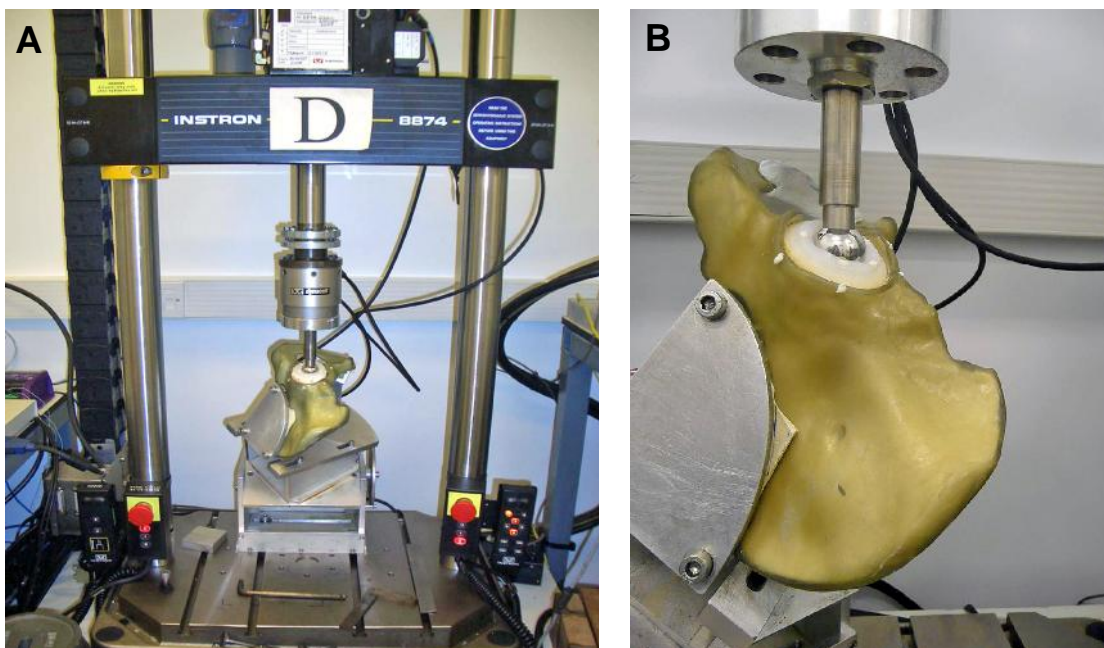
Fig 8:6 A: Hemi Pelvis repositioned under Instron actuator with 28mm V40 Head lowered into socket. B: Close up of Pelvic orientation and V40 Head engaged in socket.

During the first two experimental runs it became apparent that the sawbone model used was unable to tolerate the forces applied for the final maximal load run of 47,000 cycles at 2100N. Cracks started to appear at a stress riser just proximal to the 'Sacral' attachment of the model.
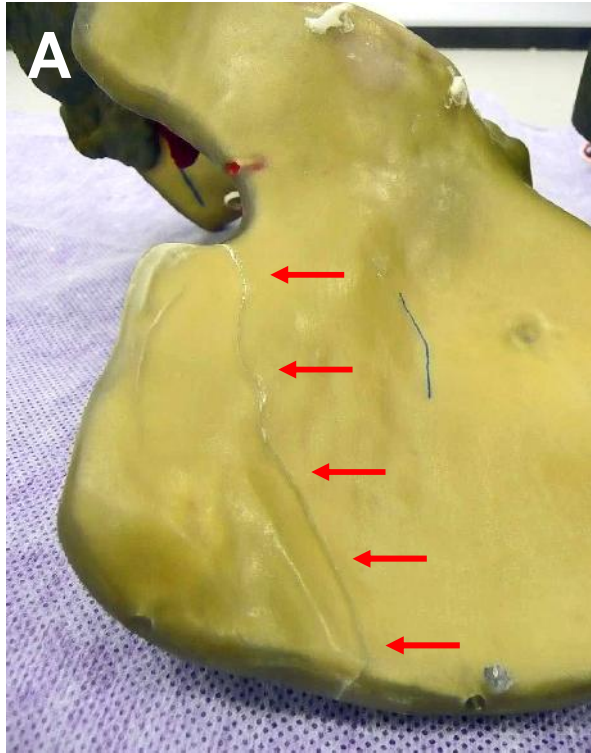
Fig 8:7 A: Hemi Pelvis model post cyclical testing showing fracture propagation across Iliac wing highlighted by arrows.

As the aim of the experiment was to assess graft stability under load and not the ultimate failure of the Sawbone hemipelvis models, the original cyclical loading protocol was modified with a final run of 48,000 cycles at a maximum load of 1600N. As this was still well within the physiological loads experienced during the normal gait cycle, therefore replicating the appropriate loads encountered in the clinical setting of revision THRs. No further failures occurred at this new loading protocol.

Sequence	Number of Cycles	Minimum Load (N)	Maximum Load (N)	Mean Load (N)	Load Amplitude (N)
1	20	50	200	125	75
2	980	50	800	425	375
3	1000	50	1200	625	575
4	48,000	50	1600	825	775

Table 8:3 New loading protocol following two failures in sawbone models at sacral attachment

8:4:9 Torque Failure test

Once the hemi pelvis model had undergone the cyclical loading testing and cup subsidence measured the final test was intended to assess the ultimate torque failure of the construct. This was again done via the instron machine with the acetabular cup attached to it via two pins located within the standard cup introducer holes within the polyethylene. Under a compressive load of 700N a rotational shear force was then applied to the cup at a rate of 0.5^{o} / sec and the force at which the bone / graft / cement interface failed was measured. As these were Left hemipelvis models the actuator was rotated in an anticlockwise direction.

Although pre programmed stop points were made to try and prevent excessive rotation, the margin between point of failure of the construct and fracture of the acetabular rim was found to be very small. This resulted in the first two samples fracturing (Fig 8:8), so due to the constraints of numbers of hemipelvis models available this section of the experiment was not continued.
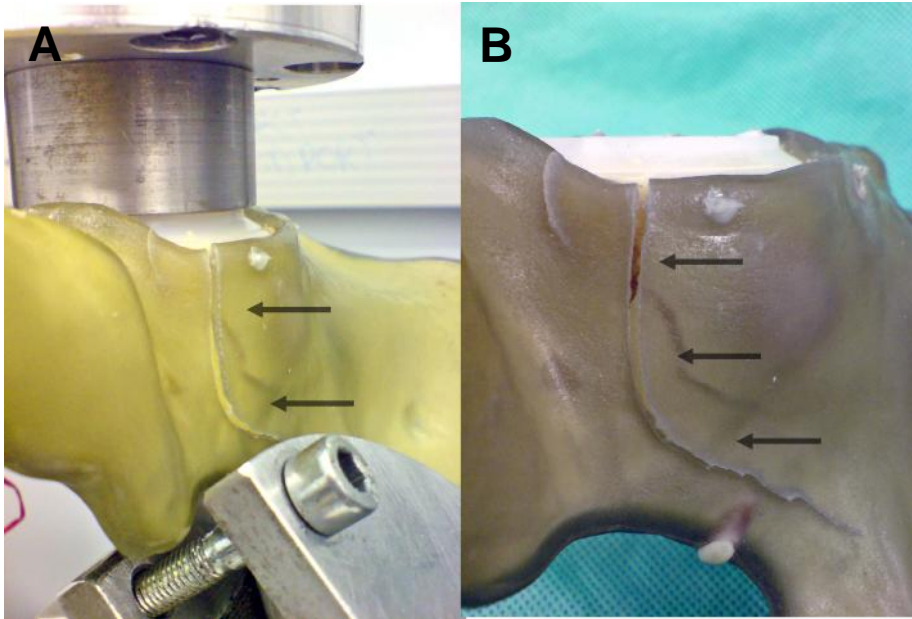
Fig 8:8 Acetabular wall fracture post tortional failure test A: In jig with coupling between cup and Instron. B: Close up of fracture line highlighted by arrows

8.5 Results

8:5:1 Validation of Penetrometer

An experienced surgeon (DGD) was analysed for 12 impaction steps with Penetrometer readings taken at each step corresponding to the 5 arbitrary zones of the graft bed (Superior 1 + 2, Medial, Pubis and Ischium See Fig 8:6). The surgeon's comment as to whether the graft was sufficiently or insufficiently compacted at each zone was noted and the corresponding penetrometer reading taken.

Surgeons assessment	Corresponding Penetrometer reading (proportional to N/m^2) Mean value +/ - SD
Insufficiently compacted	2.31 +/- 0.66 (Range 1 - 3)
Sufficiently compacted	3.67 +/- 0.52 (Range 3 - 4.75)

Table 8:4 Mean penetrometer readings from all 5 zones over 12 impactions linked with surgeon's assessment of graft stability.

From this initial experimental run it became apparent that a penetrometer reading of 3.0 was the arbitrary transition zone between what the surgeon considered insufficient to sufficiently compacted. In order to prevent any false positives it was decided that a reading of greater than 3.5 should be used to ensure sufficient compaction of all 5 areas of the graft bed at the end of the impaction process.

8:5:2 Penetrometer, Graft Weight and Subsidence Results

As described above, penetrometer readings were taken throughout the impaction process. If insufficient compaction was achieved, further graft and impaction was directed to that zone. The following table (8:5) shows the mean penetrometer readings at the end of the impaction process in the two groups.

	Standard Impaction Mean +/- SD	Vibration Impaction Mean +/- SD
Penetrometer readings	3.75 +/- 0.15	3.78 +/- 0.20

Table 8:5 Mean penetrometer readings after final impaction in control and experimental groups. p > 0.05.

The allograft used was measured both in volume, as described in the methods, and also the total weight used for each experimental run. The table below shows the mean weight of both control and vibration groups with no significant difference between the two

	Standard Impaction Mean +/- SD	Vibration Impaction Mean +/- SD
Weight of Allograft used (g).	60.1 +/- 5.20	63.6 +/- 5.2

Table 8:6 Mean weight of allograft used for each experimental run. p > 0.05

The subsidence of the cup post cyclical loading is one of the key factors as it is one of the main determinates for long term survival of the construct. The table below shows the amount of subsidence in the direction of the cyclical loading force (superomedial). There was no significant difference between the two groups (p > 0.05)

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	Standard Impaction Mean +/- SD	Vibration Impaction Mean +/- SD
Cup subsidence post cyclical testing	2.97 +/- 0.49	3.00 +/- 0.53

Table 8:7 Mean Cup subsidence post cyclical loading. p > 0.05

8:5:3 Peak stress measurements

The Graph in Fig 8:9 represents the stress imparted to the medial wall of the acetabulum for both standard control group and the Vibration group.

It shows the mean of the absolute peak stresses during impaction, and it is these maximal spikes of stress than can potentially lead to sudden fracture. It can be observed that as the graft bed builds up in the acetabulum so the stress imparted to the medial wall reduces, signified by the down sloping of the trend line. This was most marked for the standard impaction group with the vibration group giving a more linear level of stress.

The absolute maximal mean peak stress for the control group during step 1 was 110.82 (SD 17.3) MPa compared to 34.66 (SD 6.3)MPa for the Vibration group which was significantly reduced (p=0.03). There was also a significant (p<0.05) reduction for steps 3,4 and 5 as well, with step 2 p=0.06.
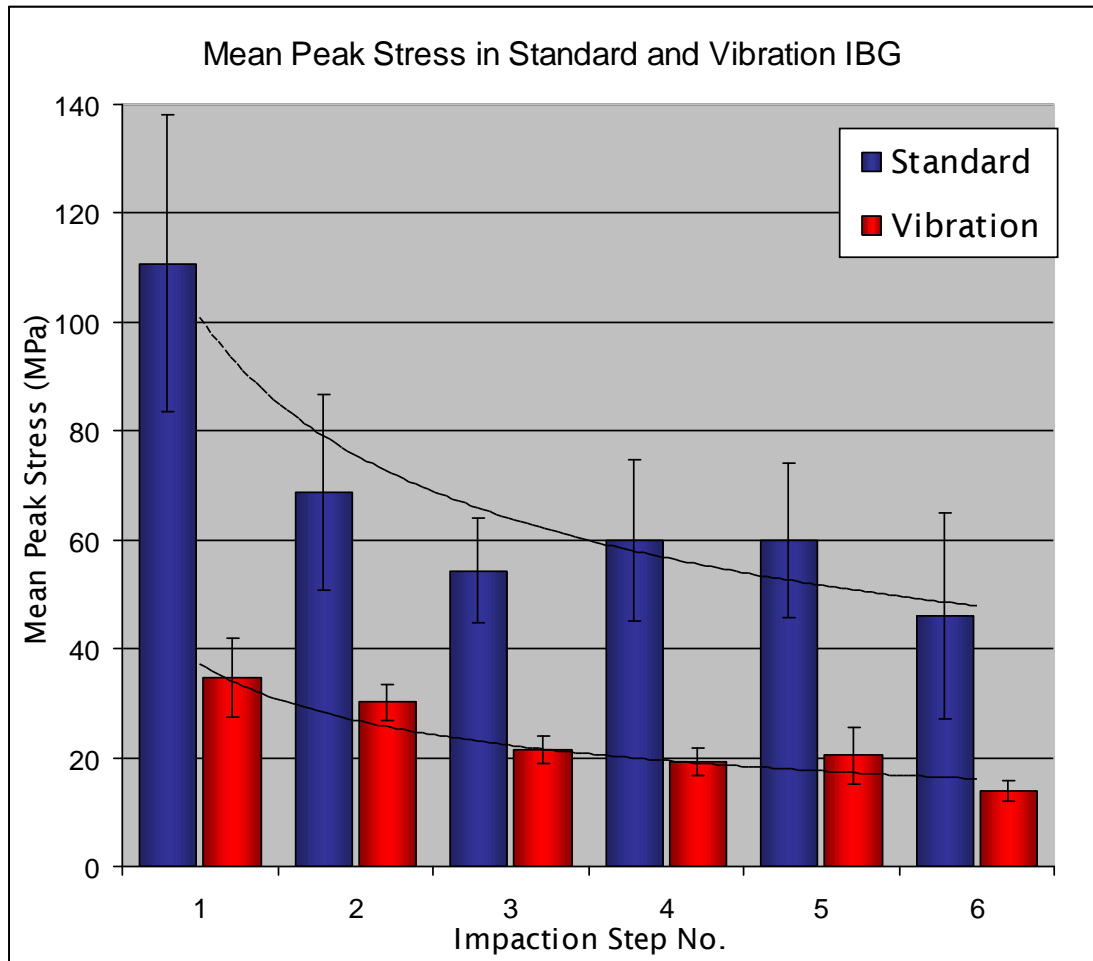
Fig 8:9 Mean Peak Stress (MPa) for Standard and Vibration impaction over the six impaction steps showing significantly less Stress in the Vibration impaction group (Red).

8:5:4 Complications

One medial wall fracture (Fig 8:10) occurred during the standard impaction process. No fractures were observed for the vibration impaction group. As previously mentioned 4 other fractures occurred during preliminary testing, but none of these were related to the actual IBG technique.
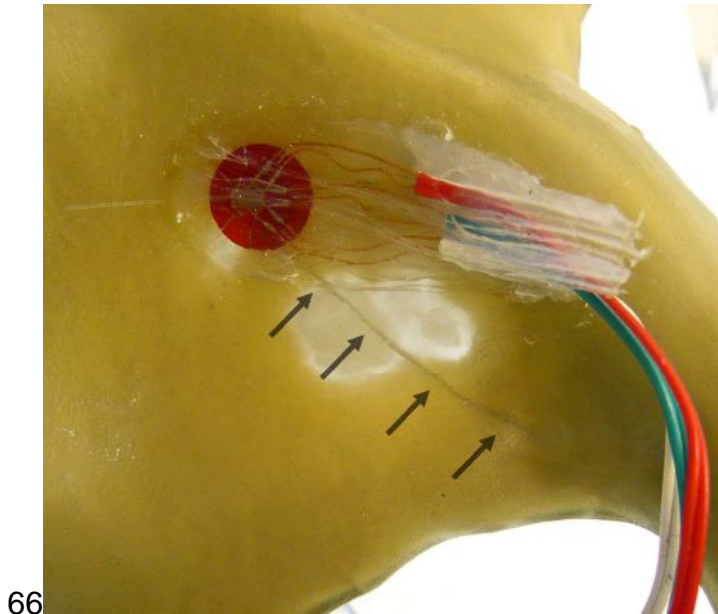
Fig 8:10 Hairline fracture through medial wall of acetabulum following standard impaction bone grafting

8:6 Discussion

The aim of this study was to assess the effect of vibration impaction and drainage compared to the current gold standard of IBG. Great care has been taken to try and eliminate as many of the main cofounding variables as possible such that Impaction forces and cup stability after cyclical loading are the two main outcome variables. All cups were implanted in the same position with no significant difference in the amount of graft used or the degree of graft compaction achieved as measured with the penetrometer.

Impaction bone grafting can be a difficult surgical skill with a significant learning curve. There a fine line between insufficient impaction and subsequent implant failure, and excessive impaction force leading to fracture and subsequent complications. Previous published studies have shown subsidence rates of between 5 and 25% (Eldridge et al. 1997). In those studies with higher failure rates, one factor could be insufficient compaction of the graft base due to concern about intraoperative fracture.

Whilst there is no substitute for the assessment and tactile feedback that experience brings when determining sufficient compaction the use of the

penetrometer in this situation has potential clinical application. Throughout the multiple experiments and impactions the readings compared to an experienced surgeon's assessment of graft compaction were very consistent. Whilst further calibration and optimisation of the design may be required, this pilot study showed potential clinical translation. This may therefore be a useful tool during the initial learning curve of an inexperienced surgeon to avoid insufficient graft compaction and subsequent massive subsidence / failure.

As the graft bed builds up the native bone is obscured from the surgeons view and hairline or non catastrophic fractures caused intraoperatively may not be noticed until the case is finished, either on post op xrays or on failure during mobilisation. This study has demonstrated that vibration assisted impaction significantly reduces the peak stress imparted to the acetabular floor thus reducing the risk of fracture whilst still achieving good graft compaction and implant stability. Despite significant reduction in the peak forces imparted to the medial wall, there was no significant difference in the subsidence after

It has also demonstrated that vibration impaction gives a much more controlled and consistent force as demonstrated by the significantly reduced variability in the peak stresses compared to the standard group.

Although the surgical technique and physiological forces involved in femoral and acetabular IBG are very different, this study has found similar benefits to the use of vibration impaction (Bolland et al 2007). In the Femoral experimental model not only were the impaction forces significantly reduced, but vibration impaction also lead to greater graft compaction and implant stability compared to standard impaction.

Subsequent to this study other groups have also analysed the effects of vibration impaction in acetabular IBG. In a study by Putzer, they used a simplified plastic acetabular cup to compare the difference in the allograft bed (Putzer D et al 2011). The variables studied were the impaction hardness, penetration resistance, contact stiffness and bulk density. 30g of allograft chips were used for each experiment. Maximal forces for the standard IBG were

369N and 308N for the vibration impaction. They demonstrated significant improvement in all the measured parameters for the VIBG group with the differences increasing with the duration of impaction. Whilst no attempt has been made to test the graft bed in the presence of a cemented cup or stability following cyclical testing, these results further support the use of VIBG for impaction grafting

Another study looking at acetabular IBG techniques by Brennan has also just been published (Brenan et al 2011). Again, using a simplified in vivo model and bovine allograft this study analysed primarily the effect of impaction technique on the graft beds shear strength and the effect of vibration frequency. The study was performed in two phases the first with a dry graft bed, and the second with a 'wet' bed with introduction of bovine blood during the impaction to reproduce the effect of back bleeding experienced clinically. They found that in the dry state vibration impaction improved the graft beds shear strength compared to standard impaction with increasing increments up to the maximum studied at 60Hz (Shear strength 342kPa vs 319kPa for standard impaction). In the 'wet' phase of the study there was no significant difference between 60Hz vibration and standard impaction. They suggest that as the saturated graft is more representative of the clinical scenario that vibration impaction is not beneficial to the IBG process due to the phenomenon of liquefaction causing disruption of the graft bed with the fluid pressure waves. As discussed earlier liquefaction is a significant consideration, and for the purposes of our study care was taken to replicate back bleeding with the addition of FCS, but to counteract the problem of fluid pressure waves disrupting the graft the impactor tamps were modified to allow drainage of the fluid. The fact that no liquefaction occurred during the vibration impaction phases despite significant graft saturation and that good implant stability was observed with extended cyclical testing suggests that this modification of the tamps is indeed a key part of the set up if the true benefits of VIBG are to be observed.

Whilst some of the early work by Bolland looked into the optimal pneumatic pressures for the woodpecker device for use in VIBG, no formal analysis of the vibration frequency was studied. The Woodpecker device has a standard

oscillating frequency of 70Hz, and as 60Hz was the maximal frequency used by Brenan et al it is difficult to know what the optimal frequency is and further experiments are required to define this. Whilst Brenan et al conclude that VIBG is not beneficial their results may have been significantly different if drainage of the tamp was undertaken, so to date the combination of these 3 acetabular and 1 femoral in vitro studies all point to a significant beneficial effect of VIBG and drainage in reducing the peak forces in IBG and improving implant stability.

8:7 Summary

I propose that the application of vibration IBG coupled with drainage for the IBG process in the acetabulum can significantly reduce the risk of intraoperative fracture whilst achieving good graft compaction and implant stability. Given the observed reproducibility of this new approach, with significantly less variability; vibration IBG offers improved 'safety margins' within the IBG process. This new approach offers the potential to reduce the learning curve for surgeons and thus offers the potential for increased adoption of the technique for replacing lost bone stock.

8:8Conclusion

Null Hypothesis

The use of vibration impaction in acetabular IBG does not reduce the forces imparted to the acetabulum.

This has been shown to be *False*

The use of vibration impaction in acetabular IBG cause insufficient graft compaction and as such risks cup subsidence

This has shown to be *False*

Chapter 9

Summary and Future Direction

9:1 Summary

The experiments carried out as part of this thesis along with the translation into clinical cases have allowed us to refute the Major Null Hypothesis. Manipulation of the SSC with extracellular membrane proteins and Hydroxyapatite have indeed enhanced the IBG's biomechanical properties, and this if used in combination with increased SSC concentration and lower impaction forces with vibration impaction should have synergistic effect to promote the formation of a new living bony construct able to support the prosthesis and host skeleton.

The conclusion from the experiments carried out in this thesis are summarised below:

The Effect of Skeletal Stem Cell Concentration on the Biomechanical Properties of Impacted Bone Graft

Following on from the seminal work by Hernigou et al on fracture non union, and preliminary studies by Tilley and Bolland on the survival and effects of SSC in IBG, this study has shown that the concentration of SSCs seeded on to IBG has a critical effect on its biomechanical properties. Failure under shear of the IBG graft bed can lead to catastrophic subsidence of the prosthesis as has been shown in a number of clinical studies with high failure rates (Eldridge et al 1997; van Haaren et al 2007). The use of a concentrated SSC fraction seeded on to the IBG therefore has the potential to improve the shear strength in vivo and reduce this risk of subsidence. From this in vitro study the concentration is above 5×10^4 cells per cc of allograft which is greater than the usual concentration found in bone marrow aspirate (Hernigou et al 2005). Further work on the optimal in vivo concentration is required and how best to achieve that concentration either by culture expansion or cell fraction separation is required.

Clinical Translation of Enriched Skeletal Stem Cells and IBG in the treatment of AVN and Fracture Non Union

The translation of the findings of SSC concentration and IBG into the treatment of clinical cases of AVN and fracture non union has shown encouraging early results. The parallel in vitro culture of the SSC and graft used for the clinical cases has also enabled detailed analysis of the biochemical and cellular changes occurring, demonstrating a significantly higher CFU-F yield and increased cellular and matrix proliferation across the allograft. Whilst there has been one failure of the AVN cases, in retrospective analysis the case may have deteriorated further than first thought at the time of treatment. Encouragingly ex vivo analysis of the retrieved femoral head has shown good structural support in the treated area with full integration of the IBG channel and normal restoration of the trabecular pattern (Aarvold et al 2010).

The Biomechanical Effect of Type I Collagen on SSC seeded IBG

This study has demonstrated than coating of the allograft granules with a layer of Type I Collagen significantly improves the Osteogenic differentiation of SSC seeded on to the scaffold potentially leading to improved new bone formation. We have also demonstrated that following in vitro culture this leads to significantly improved shear strength of the construct.

The Use of Hydroxyapatite Nanoparticles to Enhance the Biomechanical Properties of IBG

Hydroxyapatite is used extensively in Orthopaedic surgery primarily as a surface coating. This study has shown that when used in crystalline nanoparticle form it has the ability to be taken up by the SSC cells in culture and leads to significantly improved osteogenic differentiation. This study has furthered the work by Babister et al by showing that this enhanced osteogenic differentiation can improve the mechanical properties of IBG when cultured in vitro leading to significantly improved shear strength of the construct.

Murine in vivo Analysis of the Biological Effects of Type 1 Collagen Coated IBG and HAp Cultured SSC

This study used a subcutaneous murine model to analyse the in vivo effects of SSC, Type I Collagen and HAp cultured SSC. The study has shown new vessel formation around the capsule and growth into the IBG construct. Samples with SSC also showed increased cellular and matrix production between the allograft chips. There was however no demonstrable difference in the Alkaline Phospahatase activity and DNA content on biochemical assays and due to technical failures in the decalcification process further immunohistochemical analysis was not possible.

The Use of Vibration Impaction Bone Grafting as a Novel Technique in IBG

This study, following on from the work by Bolland et al on the Femur has assessed the effects of vibration impaction and drainage on an acetabular model of IBG. I have demonstrated that vibration impaction significantly reduces the peak stresses imparted to the fragile pelvis during IBG thus reducing the risk of peri-operative fracture. Despite the reduction in forces, a statistically similar level of graft compaction was achieved with no increase in the subsidence of the revision acetabular construct after physiological loading through 50,000 cycles. I have also modified a device known as a penetrometer, used in civil engineering to aid in the intra-operative assessment of graft compaction to quantify an appropriate end point and potentially aid the inexperienced surgeon.

9:2 Future Directions

The use of Skeletal Stem Cells in the treatment of musculoskeletal conditions is potentially the next big step change in Orthopaedics in the coming years. Novel ways to manipulate SSC down the appropriate stromal lineage are key factors to enable reliable, tissue specific therapies for bone or cartilage loss for example.

One of the key factors in enabling transfer of these strategies into clinical practice, particularly in the NHS is by reducing the complexity and cost of the process whilst still maintaining the beneficial outcome. Following on from my work in this thesis on the effects of SSC concentration on the biomechanical properties, our group has been collaborating with a major orthopaedic industry company designing and testing a new bone marrow aspirate concentrating system. This novel technology has won a number of awards to date and has the potential to offer a simple, cheap disposable device to allow significant SSC concentration within the sterile field of the operating theatre. This therefore has the potential to do away with the logistical and cost implications of distant SSC culture expansion as well as reducing the potential risk of contamination.

The work in this study has been with both culture expanded and centrifuge concentrated fractions of SSC both from bone marrow aspirate. No direct comparison has been made on the biomechanical outcomes between these two methods. Clarke showed that in an ulna defect model, bone marrow aspirate gave increased new bone formation than culture expanded cells (Clarke et al 2007). As discussed above there are many advantages and disadvantages of both methods and further study into the potential biomechanical differences are required. More recent interest has been looking for other potential sources of SSC for use in the treatment of fracture non union (Niemeyer et al 2010; Monaco et al 2011) in particular Adipose.derived Stem Cells (ASC). The potential benefits being a more abundant source with less donor site morbidity, more extensive in vivo analysis in this area is

required. Although there is no one surface antigen specific to the true Skeletal Stem Cell subset of the stromal cell lineage further 'purification' of samples can be achieved by flow cytometry or magnetic activated cell separation (MACS). Again no study has looked at whether these specific sub sections of the stromal cell population in the BMA confer any potential clinical biomechanical benefit.

This thesis has demonstrated the benefit of both the addition of SSC to the graft and also the reduced forces imparted by vibration impaction. It is possible that combination of these two methods would lead to less cell death due to physical disruption during the IBG process therefore leading to increased live cell concentration on the graft with enhanced biomechanical characteristics. Further study with modification of the above methods is required.

Whilst the murine model used for this study enables analysis of the samples in vivo it is clearly not a true representation of the biological conditions that IBG in the setting of revision arthroplasty is under as no load was applied following containment within the capsules.. Further work on larger animal models is required to analyse the true effects of SSC and IBG under physiological loads within a true bony cavity. A suitable sheep model has been designed for this with appropriate scaled hip hemiarthroplasty models. The true in vivo benefits of SSC concentration and extracellular matrix proteins can be analysed.

Hydroxyapatite has been used widely in orthopaedics for many years (Furlong et al 1991), but predominantly as a surface coating. The in vitro results of the HAp nanoparticles are very encouraging, but clearly before this can be translated into clinical practice, further work on the in vivo effects are required. Studies to assess the local rate of bone formation and cessation of formation, and on any potential side effects either at a cellular level locally and on potential distant spread to other tissues would need to be performed. Whilst I have demonstrated uptake into the cells with and excess of the nanoparticles removed during the washing process, further work to exclude any potential mechanical problems of bearing surface wear are required.
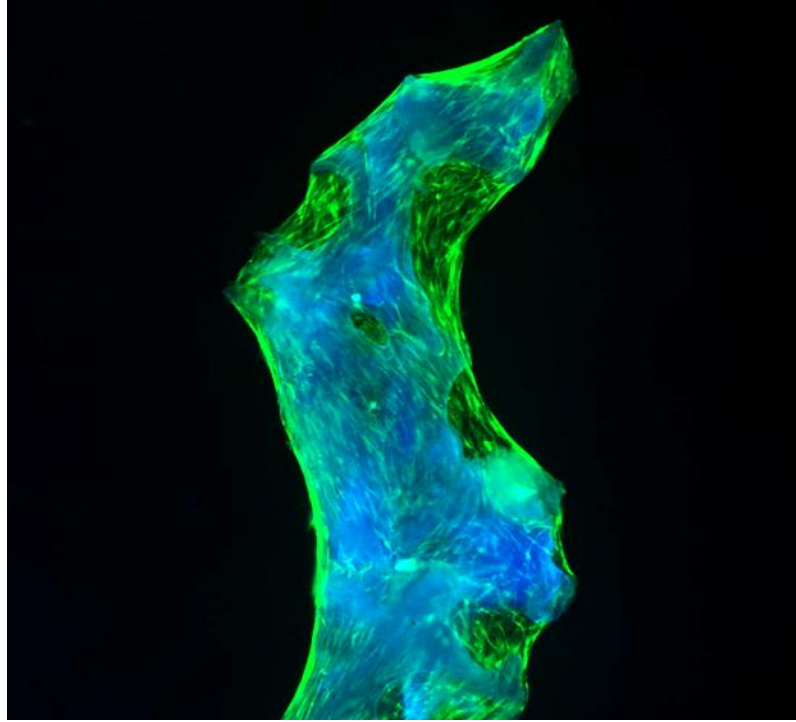
Fig 9:1 Cultured SSC on Allograft Scaffold

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