REGENERATION OF NEW BONE IN REVISION HIP REPLACEMENTS USING A TISSUE ENGINEERING TECHNIQUE

By

MICHELLE KORDA

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOMEDICAL ENGINEERING
UNIVERSITY COLLEGE LONDON

JUNE 2007

THE CENTRE FOR BIOMEDICAL ENGINEERING
INSTITUTE OF ORTHOPAEDICS AND MUSCULO-SKELETAL SCIENCE
UNIVERSITY COLLEGE LONDON
ROYAL NATIONAL ORTHOPAEDIC HOSPITAL TRUST
STANMORE
MIDDLESEX
HA7 4LP
UNITED KINGDOM

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Impaction allografting is used to fill osteolytic defects during revision total hip replacements (rTHR). However clinical results are inconsistent as bone fracture and excessive implant migration are a major complication. Most importantly allograft does not adequately enhance new bone formation. As a consequence the results of rTHR are often inferior to primary THR due to lack of bone stock. Other studies indicate that tissue engineering (TE) using Mesenchymal Stromal Cells (MSCs) seeded onto a resorbable scaffold can regenerate new bone.

The overall aim of my thesis was to test the hypothesis that TE of autologous MSCs and MSC derived osteoblast cells incorporated with impaction allografting will significantly enhance new bone formation in revision THRs. The study is divided into *in vitro* and *in vivo* phases. In the *in vitro* phase the technique of osteogenic differentiation and seeding of the autologous ovine MSCs onto allograft was optimised. Previously determined normal impaction forces of 3, 6 and 9kN were used to study the viability of these impacted MSCs and osteogenically seeded cells on the allograft. The results showed that both MSCs and osteoblast cells are affected by the impaction forces. However the MSCs can survive normal impaction forces of 3-6kN while osteoblast cells can only survive impaction forces under 3kN.

The *in vivo* phase was an ovine model used to compare new bone formation between MSCs, osteoblasts and a control group in an orthotopic, ectopic and revision hip. The results show that overall MSCs enhance new bone formation in all the scaffolds compared with the osteoblast and control group. However osteoblast cells do not contribute to new bone formation. The conclusion from the study was that MSCs in conjunction with the impaction allografting technique enhance new bone formation. The tissue engineering technique can be developed into a clinical application in revision THRs where large bone defects have become problematic.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr Jia Hua for disseminating his vast knowledge to me in a critical, philosophical and practical manner. His patience and good humour have contributed to my PhD experience being thoroughly exciting and enjoyable and I could not have wished for a better supervisor.

It is not possible to thank Professor Blunn enough for allowing me to work in such a vibrant and inspirational department. I will always remember his support and encouragement throughout my thesis and if I have become a better scientist, much of the credit is due to him.

Thank you to everybody at the Institute of Orthopaedics for being friends as well as colleagues particularly Rebecca Porter for her cell knowledge. In addition thanks to all those at the Royal Veterinary College at North Mymms. Gillian Hughes, you are the purveyor of fine foods and the ultimate anaesthetist. Thank you.

The following people have contributed to the completion of this thesis and my emotional wellbeing throughout in varying degrees. The Zimmer lab has been a wonderful place to work, thanks to everybody there who has helped me. Thank you to Kirsty Phipps, Isabel Tullock, Philippa Rust, Shankar Balaratnam, Nick Little. Nima Heidari, thanks for your intelligent contribution and nifty suturing; Annie Bartram for improving my spelling and grammar; Keith, Mark and Bob thanks for making all those tubes! Louisa Collins, thanks for rescuing my bones. Siva Sundar, thanks for always having a spare pen/camera/charger/USB cable/memory stick and a stash of biscuits. Priya Kalia, thanks for being a good friend.

Finally, thanks to friends and family. Thanks to the lovely Helen Brown. Thanks to Mum, Dad and Serena for caring about and listening to presentations, sometimes the same ones repeatedly and at increasing speed, even during Coronation Street!

LIST OF FIGURES

4.10	**a** Image of the middle region of the allograft ctrl group. The paragon has stained new bone (NB) bright pink. The allograft (AG) remained light in colour. Active osteoblasts (OB) can be seen laying down osteoid, which stains lighter in colour on the surface of new bone. **b** During intramembranous ossification (IO) the osteoblasts become enclosed in the mineralizing matrix becoming osteocytes. In this image osteocytes are relatively flattened, darkly stained specks. The Howship's Lacunae (HL) are depressions in the bone resorbed by osteoclasts that have preceded new bone formation. New bone is associated with angiogenesis; the marrow cavity (MC) contains blood vessels (BV).	**124**
	c Image taken at the middle region of allograft MSC group. The MSC group shows a similar morphology to the control group with NB forming in contact with remaining AG. There is also fibrous tissue filling all the gaps not occupied by new bone, allograft or other elements of mesenchymal tissue.	**125**
4.11	The graph represents percentage new bone formation at the middle and host-graft interface (±SE) after six weeks *in vivo* after impaction at 3kN	**126**
4.12	The graph represents percentage remaining allograft at the middle and interface (±SE) after six weeks *in vivo* The area of HA occupying the microscopic field at time zero was 49%.	**126**

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 Background

Bone and joint diseases are among the most common conditions affecting health and quality of life in the UK. For severe osteoarthritis of the hip joint, total hip replacement (THR) is one of the most successful procedures for improving quality of life. The survival of the implant is effected, amongst other things, by the age and the activity level of the patient (Birrell *et al.* 1999). In the UK approximately 43,000 hip replacements are inserted every year (Bourn 2003). According to the National Joint Registry for England and Wales the overall revision rate in 2003 was 9.3% (van der Meulin *et al.* 2004). Reasons for revision include loosening due to poor initial fixation (Amstutz *et al.* 1992), stem fracture, recurrent dislocations (Franklin *et al.* 2003) and infection (Masterson *et al.* 1997). Aseptic loosening as a result of osteolysis is the predominant cause of failure of THRs in both cemented and uncemented cups and stems (Harris 1995; Cooper *et al.* 1992). Articulation of implant surfaces leads to the release of wear debris resulting in local inflammation, osteolysis and dislocation (Harris 1995; Cooper *et al.* 1992; Amstutz *et al.* 1992).

Bone is a regenerative material that, under homeostatic conditions, undergoes a continual cycle of resorption and mineralisation. The normal balance of this cycle is disrupted by osteolysis. The lack of bone stock caused by osteolysis of bone surrounding the primary implant effects the fixation of the revision implant (Masterson *et al.* 1997). As a consequence, the results for revision hip replacements are inferior compared to primary hip replacements. This was reflected by a more rapid increase in revision operations compared with primary operations (154% and 18% increase in procedure rates repectively) which rose from 1 in 12 in 1991 to 1 in 5 in 2000 (Dixon *et al.* 2003). Subsequent re-revisions can lead to a cumulative depletion in bone stock that would normally provide stability for the implant (Krishnanmurthy *et al.* 1997; Sporer and Paprosky 2004; Eldridge *et al.* 1997). In cemented applications, fixation has been improved by developing better methods of cementing the hip in place. The first generation technique included mixing the cement by hand and manual insertion with little or no canal preparation. Techniques developed for the second generation involved

preparing the canal by brushing and pulsatile irrigation, but still with a manual technique. A third generation technique involved cement void reduction by vacuum or centrifuge mixing which leads to improvements in tensile strength, cement gun injection by pressurisation, which allows the cement to intrude more easily into the bone, and pulsatile lavage of the femoral cavity (Wright and Li 2000; DiMaio 2002).

The problem of osseous integration of host bone with the implant in uncemented applications has been addressed by the introduction of porous implant coatings of titanium (Stephenson *et al.* 2003; Soballe *et al.* 1992), or cobalt chromium (Jacobs *et al.* 2000). The suggestion is that bone interdigitates with the pores of the implant surface resulting in more permanent fixation. Adversely, a porous coated stem has a higher surface area for metal particle release (Jacobs *et al.* 2000). Although metal particles are less implicated in osteolysis than ultra high molecular weight polyethylene (UHMWPE) (Shanbhag *et al.* 1993), they do contribute to increased wear of polyethylene liners (Amstutz *et al.* 1992). It could be argued that hydroxyapatite (HA) coatings are more successful at improving bone ingrowth into implants (Stephenson *et al.* 2003) whilst sealing the bone-implant interface, preventing migration of wear debris (Coathup *et al.* 2005). However, reports on HA coatings have been inconsistent. In some studies HA has been optimal in providing anchorage and integration, but on closer examination the apposition of the implant was with fibrous and soft tissue (Buma and Gardeniers 1995) or fibrocartilagenous tissue (Soballe *et al.* 1992). The problems associated with fixation are exacerbated in revision situations where bone stock is reduced.

Aim

To enhance new bone formation around revision THRs by incorporating mesenchymal stromal cells (MSCs) with allograft used for impaction grafting.

Hypothesis

Incorporating MSCs into allograft or HA granules which are then impacted, will increase the rate and extent of bone formation.

1.2 The hip joint

1.2.1 Anatomy and pathology of the hip joint

Improvement in hip function and pain relief are the two main reasons for hip surgery. Most commonly THR is performed to relieve pain and restore function which is affected by diseases such as osteoarthritis, rheumatoid arthritis, trauma, osteonecrosis and ankylosing spondylitis. The hip joint constitutes a ball and socket anatomy. The ball, known as the femoral head, is the proximal extension of the thighbone which fits closely into the socket, a depression in the pelvic bone called the acetabulum. The aim of a total hip replacement (THR) is to remove the diseased or deformed and damaged hip joint and replace it with an artificial hip, resulting in resilience and range of movement as close as possible to that of a fully functioning natural hip joint. The nature of the condition requiring treatment and the age and activity level of the patient will determine the suitability of the surgical approach used to achieve the optimal outcome for the patient. In some instances only hemiarthroplasty may be required. In this case the surgeon replaces only the femoral head and leaves the acetabulum intact. This is used for patients where circulation to the femoral head has failed, due to dislocation or fracture, and the femoral head has died.

1.2.1.1 Bone architecture, physiology and function

Bone is a hard, vascularised and innervated tissue that comprises the mammalian skeleton. The skeleton provides mechanical support, where bones act as a lever to which muscles are attached for locomotion; it protects the internal organs of the body, such as the brain and spinal cord, and maintains calcium homeostasis via hormonal control. Calcium release from the bone into the blood is under the control of the parathyroid hormone (PTH). Conversley, hormones interact with cellular receptors in feedback loops, allowing calcium to be taken from the blood and incorporated into bone (Hancox 1972). Another protective role of the endoskeleton is that it contains the marrow, which is the origin of the haematopoeitic system.

The external surface of a typical long bone is surrounded by the periosteum, consisting of a fibrous layer and inner cambial layer which supplies blood and lymphatic vessels, as well as nerves, to the bone. This encases cortical or compact bone, which is comprised of concentric cylinders of lamellae called osteons. At the centre is a Harvasian canal through which the blood vessels, lymphatic vessels and nerves pass. Capillaries also communicate between Harvasian systems through Volkmann's canals. Between the osteons are interstitial lamellae, which are the remains of older, remodelled Harvasian systems. Cortical bone makes up 80% of the skeleton and has a slow turnover rate. Situated between the lamellae are osteoblast derived cells that, once having become trapped in small cavities called lacunae, change in phenotype and are termed osteocytes. The cortical bone is the outer casing which contains cancellous or trabecular bone, made of interconnected struts called trabeculae (figure 1.1). The struts interspersed with a series of voids allow the bone to be strong as well as light without adding mass. Therefore the structure of bone is efficient as it provides the maximum strength for the minimum weight. The hollow geometry of the long bone resists bending loads because most cortical bone lies far from the neutral axis of the cross section of the bone. This gives it a high moment of inertia of an area, which is resistant to bending. As well as playing a biomechanical role, the central canal has a physiological function as an unstressed cavity, that houses a marrow reservoir and a medullary blood supply (Bostrom *et al.* 2000).

Four cell types contribute to bone formation and remodelling: osteoblasts, osteoclasts, osteocytes and bone lining cells. The osteoblast cell is mainly responsible for laying down the organic matrix of the bone in the form of osteoid, which is calcified soon after deposition. This is coupled to bone resorbtion by osteoclasts. The osteoclast is a multinucleated, phagocytic cell involved in the resorption of bone during remodelling. The osteocyte originates from the osteoblast as described above, but is less active in matrix production and is more likely to behave in a communicating role with other osteocytes through its long processes. Osteocytes may be involved in the transduction of mechanical signals to other more active cells and certainly have a role in calcium homeostasis. Bone lining cells are osteoblasts that cover the surface of all bones. The functional activity of the cell depends on the structure. Tall, plump cells are metabolically

active regulating bone osteoid production. Elongated, flat cells are quiescent bone lining cells that are involved in the process of bone matrix degradation (Bostrom *et al.* 2000).

Smaller voids on the bone are also filled with marrow. The structure of bone reflects its function as a dynamic organ. The surface area to volume ratio of trabecular bone allows interplay of resorption and mineralisation required for the constant remodelling of bone that occurs throughout life.

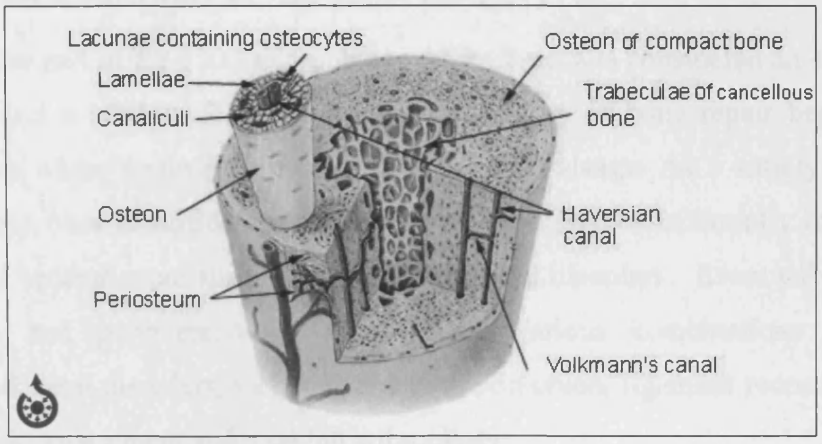


Figure 1.1 Long bone physiology (from Wikipedia: The Free Encyclopaedia http://en.wikipedia.org/wiki/Osseous_tissue)

Organic and inorganic components of bone

Bone development is either by endochondral or intramembranous ossification. Endochondral ossification is the process whereby cartilage is calcified forming trabecular bone. Intramembranous ossification is where mineral is deposited on a membrane such as the periosteum. Remodelling involves mineral being laid down onto bone by osteoblasts and removed by cells called osteoclasts. This process is controlled by hormones, local cytokines and by mechanical loads.

The organic matrix of the bone is mainly made of type I collagen (collagen I). Collagen comprises 90% of the organic matrix with osteocalcin (OC), osteonectin (ON), osteopontin (OP), and proteoglycans contributing 10%. Essentially these proteins have a role in the bone orientation and structure. The inorganic component of bone reinforces the organic matrix, making it strong and rigid. Mineralisation of bone occurs after the osteoblasts have secreted osteoid in the

form of collagen. Matrix vesicles are contiguously secreted and contain the enzyme Alkaline phosphatase (ALP). ALP encourages phosphate and calcium ions to accumulate between collagen fibrils in 'hole zones'. Eventually calcium hydroxyapatite (HA) crystals precipitate (Young and Heath 2000). Hydroxypatite, the inorganic component of bone, has the general formula $Ca_{10}(PO)_4(OH)_2$. Knowledge of the chemistry of HA is important in creating new synthetic materials that are bioactive as well as biocompatible.

1.2.2 The evolution of hip joint surgery

In the latter part of the 1700's amputation of the limb was considered an expedient solution and a panacea for all injuries. The history of bone repair began with techniques where treatment was restricted to limb salvage. As a variety of bone pathologies were identified and technology and materials concurrently improved, treatment became more specific to musculoskeletal disorders. Eventually metals, ceramics and polymers were employed in various combinations to treat musculoskeletal disorders, including fracture, non-union, ligament reconstruction, spinal disk replacement and total hip arthroplasty.

Henry Park (1744-1831) developed the technique of joint excision, which is the removal of 'all the bones which form the joints', thereby creating a callus that replaced the diseased parts (Gomez and Morcuende 2005). Joint excision was widely practiced, regardless of the etiology of the joint disease being treated. The first example of reparative hip surgery in the form of exision arthroplasty is attributed to Anthony White of Westminster Hopital in 1822. He excised the upper end of the femur in a nine year old boy for sepsis and deformity, restoring mobility and relieving pain but compromising stability (Newman 1971). Girdlestone (Gomez and Morcuende 2005) was the first to use the excision technique for degenerative disease. Further surgical developments included interpositional arthroplasty to prevent recurrence of ankylosis. This was pioneered by Ollier and others where soft tissue was inserted between the joint surfaces (Gomez and Morcuende 2005). Other materials placed between bone surfaces ranged from pig bladder (William Steven Baer at the Johns Hopkins Hospital), to gold foil (Robert Jones) (Newman 1971).

1.2.2.1 Artificial Joints

The earliest pioneer in total hip replacement is widely recognised as Themistocles Gluck who, in 1891, constructed an ivory ball and socket joint which was fixed to the bone with nickel plated screws (Newman 1971). The materials were refined using Wiles' design in 1938 of a stainless steel head and acetabulum and also by the Judet brothers who used acrylic prostheses in 1948, improving fit and simplifying the operation (Newman 1971). Other refinements of prostheses followed, including the use of dental acrylic cement for implant fixation, cobalt chrome components by McKee and Peter Ring's cementless implants, which had metal on metal components. However, the biggest landmark in total hip replacement came with the development in the 1960s of the low friction arthroplasty concept by Sir John Charnley (Newman 1971).

After first using polytetrafluoroethylene (PTFE), Charnley used the relatively low friction couple between the high density polyethylene for the acetabulum and stainless steel for the femoral head components. The use of these materials was a major factor in reducing wear. His implants were also designed to have small metal heads, 22mm in diameter, conferring less torque whilst providing a thick plastic cup. Ever since Charnley pioneered the use of metal on polyethelene components in hip replacements in the 1960s, THRs have provided the most expedient solution to joint pain as a result of severe osteoarthritis.

1.2.3 Primary Total Hip Replacement

A thin layer of cartilage covering the articulating surfaces of the femoral head and the acetabulum allows them to glide against one another. In the operation the diseased parts of the hip joint are removed, including cartilage covering the acetabulum and parts of the femur that may occlude access to the femoral shaft. The acetabulum is reamed to receive a prosthetic component composed of a polyethylene liner that is either cemented directly into the socket or is metal backed, which allows the implant to be fixed using cement or by direct bone apposition. For the femoral component, the intramedullary canal is prepared using rasps which re-shape the canal to receive a metal stem that is either cemented or press fit into place. A metal or ceramic head, that replaces the ball of the joint, is then fitted onto the stem (Figure 1.2). The possible combinations of bearing

surfaces, in decreasing rates of volumetric wear, are: metal on polyethylene, such as a cobalt-chrome head with ultra high molecular weight polyethylene (UHMWPE) or more recently cross linked polyethelene liners; metal-on-metal, for instance a cobalt-chrome femoral head coupled with a cobalt-chrome liner, or ceramic-on-ceramic combinations of head and liner (Heisel *et al.* 2003; Park *et al.* 1995). In the UK the most common bearing combination is a cemented UHMWPE acetabular cup articulating with cobalt-chrome, whereas in the USA the metal femoral head articulates against a cementless UHMWPE cup.

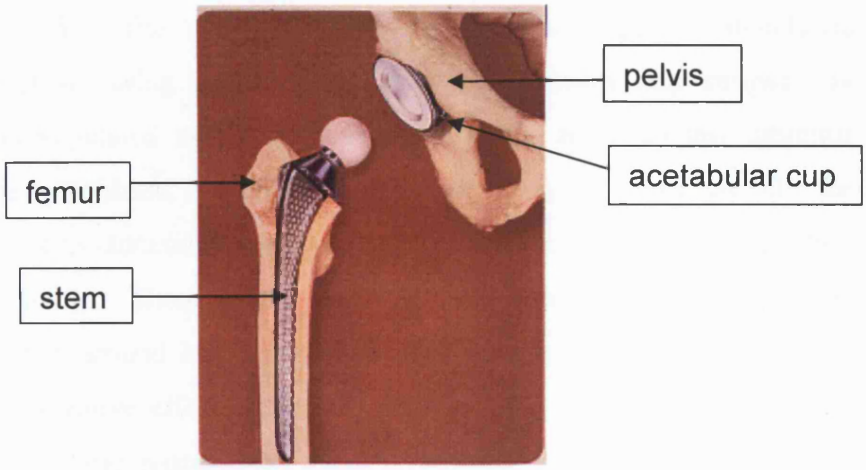


Figure 1.2 Total Hip Replacement (from http://www.materials.qmul.ac.casstud/implants)

1.2.4 Osteolysis in the failure of THRs

Wear particle induced osteolysis often results in a decrease in the bone stock which is required for implant fixation in revision hip replacements (Harris 1995; Cooper *et al.* 1992). The mechanism of osteolysis is triggered by particles of metal, cement or polyethylene that are released when surfaces of the implant articulate with one another. These particles are able to travel along small spaces between the implant interfaces, such as the stem-cement interface. When they come into contact with biological tissues these particles become engulfed by inflammatory cells, such as macrophages and giant cells. This activates macrophages which release bone resorbing mediators such as interleukin-1 (IL-1) and tumour necrosis factor (TNF-α), an osteoclast activating cytokine stimulating osteoclasts to differentiate from monocytes and resorb bone (Athanasou 1996). There is evidence that macrophages contribute directly to osteolysis by differentiation into

osteoclasts. Macrophages have been isolated from periprosthetic tissues and differentiated into osteoclasts *in vitro* (Sabokbar *et al.* 1997). The RANK-RANKL-OPG pathway constitutes a basic control network that regulates bone remodelling. This is also the case in osteolysis where bone cells such as osteoblasts and osteoclasts regulate bone turnover. The transmembrane receptor, Receptor Activator of Nuclear factor kappa B ligand (RANKL), is expressed on the surface of preosteoblastic cells which binds to (Receptor Activator of Nuclear factor kappa B (RANK) on osteoclast precursors. This allows differentiation and activation of cells of the macrophage/monocyte lineage into osteoclasts and thus leads to resorption. The ability of pre-osteoblasts to support osteoclastic development is lost during differentiation down the osteoblastic pathway as RANKL is downregulated and osteoprotegerin (OPG), an osteoclast inhibitor produced by the osteoblasts, is increased. It would be counterproductive for the mature osteoblasts to stimulate osteoclasts to destroy osteoid they have just laid down (Khosla 2001). There is also evidence that osteolysis is triggered by hydrostatic pressure around hip components after hip replacements. In a study comparing the resorptive effect of cement particles and pressure in a rat tibial diaphysis, the osteolytic process was more greatly influenced by biomechanical stimuli than the cement particles (Björn and Aspenberg 2003).

The most aggressive form of osteolysis is described clinically as erosive inflamed bone resorption, where a fibrous membrane eventually surrounds the loosened implant. This can be observed in cemented acetabular components. Figure 1.3 shows periprosthetic osteolysis of a cemented femoral component in a male patient. The front view (figure 1.3a) displays gross osteolysis on the medial aspect, showing aseptic loosening between the cement and the bone. The lateral view (figure 1.3b) shows localised osteolysis on the distal stem on the anterior side. However, periprosthetic osteolysis is also prevalent in cementless femoral components despite excellent femoral fixation at five years (Cooper *et al.* 1992). This indicates that despite improvements in fixation techniques and optimisation of surface characteristics, such as implant coatings of HA (Coathup *et al.* 2005), there is still debris release due to wear (Amstutz *et al.* 1992). Although it may be possible to minimise wear debris release, it is likely that bone stock will be depleted by osteolysis for the foreseeable future.

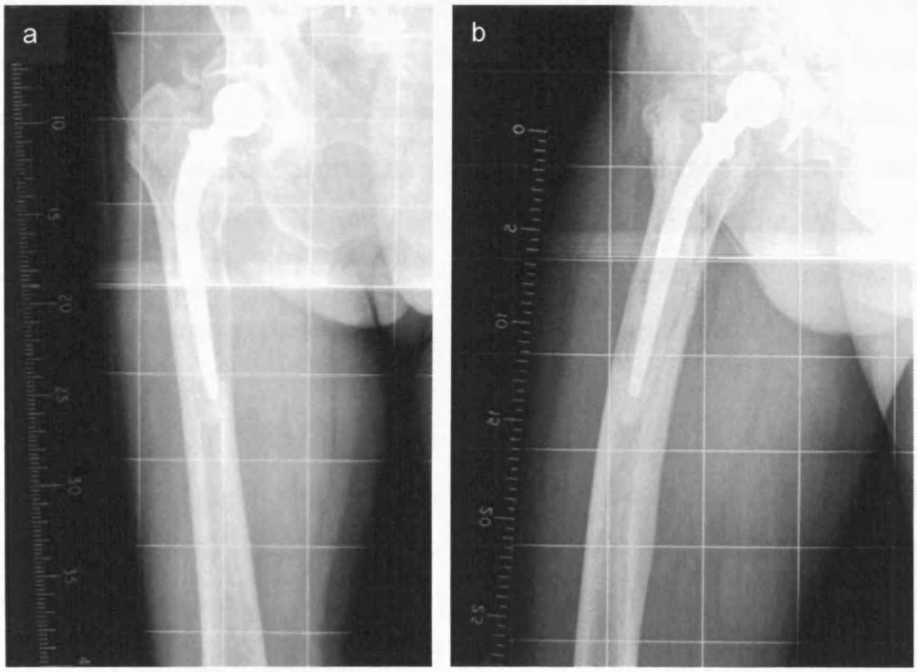


Figure 1.3 Radiograph showing periprosthetic osteolysis, **a** AP view, **b** lateral view

1.3 Revision THR

The purpose of revision total hip replacement is to replace the previously loosened stem and restore function. However, often there is not sufficient bone stock available for fixation of the revision stem. Provided the implant is mechanically stable, the load transferred onto the remaining bone should stimulate bone regeneration. In the past, revision THRs have used the cemented stem or an extensively porous coated uncemented stem i.e titanium plasma sprayed with HA (Nelissen *et al.* 1995). In these situations the femoral stem often by-passes the proximal osteolytic lesions and is fixed more distally than in primary hip replacement. Unfortunately, bone stock is not only depleted by osteolysis but further loss of bone is incurred when the primary implant is removed during revision surgery, resulting in an additional challenge for the surgeon in reconstituting bone stock.

1.3.1 Impaction grafting

One of the techniques in use today is implantation of bone graft, which is impacted into the endosteal cavity to create a neo-medullary canal in the femoral shaft. Debridement of the femoral canal involves the removal of the loose prosthesis,

including all cement, debris, granulomata and fibrous membrane. If there are cortical defects, the graft is constrained within the femoral canal using a mesh with wire cerclage. The neo-medullary canal is shaped by a series of tapers that allow the graft to be packed under force against the walls of the femoral canal and then the cemented component is introduced (Gie *et al.* 1992; Gie *et al.* 1993). Impaction of morsellised cancellous graft is widely accepted in clinical practice as the best way to reconstitute bone stock from a mechanical, as well as a physiological approach.

Impaction grafting, predominantly combined with the Exeter X Change system developed by Slooff, Gie and Ling (Ling 1997), goes some way towards addressing the requirements of immediate fixation and reconstitution of the bone stock at revision operations. In the 'Slooff and Ling technique' impacted morsellised graft provides a firm mantle for the femoral implant (Tägil 2000; Leopold *et al.* 1999) which is cemented in place. Originally autograft had been used to correct acetabular protrusion in rheumatoid arthritis, as it was found to be an improvement compared to wire mesh or oversized acetabular cups (Leopold *et al.* 2000). Slooff modified the technique by impacting allograft instead of autograft into osteolytic defects of the acetabulum (Sloof *et al.* 1984). Ling developed the technique further by using allograft in femoral reconstruction (Gie *et al.* 1993). Impaction grafting is now widely used worldwide for reconstructive surgery for fractures, limb salvage, spinal reconstruction and THR.

1.3.2 Autograft

Bone is the most frequently transplanted tissue in humans after blood, with 200,000 bone transplants performed yearly in the United States. The first recorded successful bone graft was by Job Van Meek'ren in 1668 (Lane and Sandhu 1987). Autograft is boney tissue that has been removed from a non-essential part of the skeleton e.g. the iliac crest, and transferred to another location in the same individual to repair bones essential for weight bearing (Niklason 2000). There is undoubted histocompatibility of autograft (Burchardt 1987). Autograft is the safest method of bone transplant as it involves a closed surgical system. Bone merely requires transport from one site on the patient to another, so there is less chance of

cross contamination, disease transmission or viral infection than there would be with allograft.

Autograft is widely accepted as the 'gold standard' for bone transplant due to its osteogenic, osteoinductive and osteoconductive properties. Osteogenesis is bone regeneration by pre-existing differentiated or determined bone forming cells from either the graft or the host (Lane and Sandhu 1987). The morsellised autograft may contain active osteoblasts or osteoprogenitors from the periosteum or marrow. As the autograft is freshly harvested, this means that the host's own viable cells will contribute to osteogenesis. However, it is debatable whether osteocytes trapped in bone lacunae are active or would be able to free themselves to contribute to the surrounding environment for osteogenesis (Andrew and Bassett 1972). The autograft would also act as a mechanical support for bone formation and encourage osteoconduction. Osteoconduction is the formation of bone conducted along the graft surface. Cancellous graft is ideal for this purpose as it is composed of a large surface area (Khan *et al.* 2005). The possibility that autograft, if handled correctly, will contain a reservoir of bioactive substances as well as cells means that it will also be osteoinductive. Osteoinduction can be defined as a process whereby the donor tissue causes the host's mesenchymal stem cells to differentiate into osteoblasts, producing bone (Logeart-Avramoglou *et al.* 2004).

There are many drawbacks to autologous grafting. An additional cost is associated with harvesting autologous bone (Khan *et al.* 2005). The invasive procedure involved in autologous harvesting can cause unnecessary pain, haemorrhage, nerve damage, risk of bacterial infection, muscle weakness, nerve injury and blood loss. The associated pain from a combination of damage is referred to as donor site morbidity (Niklason 2000). There is also a limited bone supply, particularly for large defects and defects in children. The ability to form a functional shape from this tissue is also compromised (Lane and Sandhu 1987). Therefore allografts have become the most common alternative to autografts and are used in revision techniques such as the Exeter X Change System described above.

1.3.3 Allograft

Allograft is bony tissue transplanted from one individual to another of the same species that is genetically disimilar (Burchardt 1987). It supplies a mechanical function by supporting the implant when subjected to load. Although allograft is less osteoinductive than autograft, as deactivation of cells may have I from irradiation, it is still osteoconductive. Allograft provides a scaffold for the ingrowth of blood vessels and new bone and a surface for cell migration from the host matrix.

Tissue banks prepare, screen and store allograft, in a function akin to modern blood banks. Bone tissue is harvested from cadavers, or living donors e.g. amputees, femoral neck fractures (Tomford *et al.* 1987). Allograft is commonly sourced from the cancellous bone of femoral heads removed during primary THR. This can be stored locally in regional bone banks. The bone is subsequently morsellised by passing through a bone mill. The resulting bone chips can be used directly or preserved by either freezing (Gie *et al.* 1993), or freeze drying under aseptic conditions. It may also be sterilised using gamma irradiation. The relative advantages of long term storage of freeze dried graft is outweighed by the suggestion that it's mechanical properties are adversely affected (Khan *et al.* 2005). The incorporation of both allograft and autograft is similar to the process of fracture healing, with autograft incorporation proceeding more quickly than allograft incorporation (Ullmark and Obrant 2002; Burchardt 1987). There is evidence of remodelling of bone graft after revision surgery in the femur with osteocalstic resorption of the surface of graft granules and simultaneous deposition of osteoid and bone (Linder 2000).

Essentially bone remodels as a response to mechanical loading according to Wolff's law: 'Remodelling of bone ... occurs in response to physical stresses – or to the lack of them – in that bone is deposited in sites subjected to stress and is resorbed from sites where there is little stress' (Andrew and Bassett 1972). Load is not the only impetus for new bone formation, but it is the predominant signal for directing and maintaining bone formation in sites of depleted bone stock. Bone responds to injury in three phases: inflammation, repair in the form of soft callus

then hard callus, and remodelling. During the inflammation phase the graft is invaded by cells as a response to haemorrhage. Motion between graft particles and host is minimised by a bridging of soft callus, consisting of osteoblasts and eventually a callus of woven bone. This is accompanied by vascular invasion of the graft from host tissue. The graft facilitates the migration of bone forming components, termed 'creeping substitution', because of its biomechanical properties (Khan *et al.* 2005; Lane and Sandhu 1987; Andrew and Bassett 1972).

The most emphatic reason against the use of allograft is the potential for transmission of viral pathogens such as HIV. Musculoskeletal allografting has resulted in two reported cases of HIV infection since 1980 (Khan *et al.* 2005). However tissue banks now adhere to stringent pre-screening including serological tests for transmittable diseases such as HIV, Hepatitis B and Hepatits C (Buck and Malinin 1994). Bacterial and some viral contamination can be overcome by sterilisation methods such as gamma irradiation or ethylene oxide treatments. Although sterilisation can reduce the risk of infection, there is still risk of resilient HIV (Buck and Malinin 1994) or infection through careless handling. This may have been the scenario in the fatal case of *Clostridium* species infection as a result of allografting (Khan *et al.* 2005). Sterilisation by gamma irradiation can also prevent an immunogenic response. The possibility of rejection of the allograft is high unless the antigenic components of the graft are removed by a series of washing steps then irradiation. The processing techniques ultimately compromise the mechanical strength of the graft to some degree (Khan *et al.* 2005; Pelker and Friedlaender 1987). Additionally, processing in tissue banks means that allograft production is more expensive and time consuming than obtaining autograft. As the tissue bank relies on continual donation of graft, there is a finite supply. Due to the varying ages of the donors, combined with their disease status (e.g. osteoporosis), the allograft will also be of inconsistent quality.

Impacted cancellous allograft has been widely used as a bone stock replacement with and without cement (Ling *et al.* 1993; Gie *et al.* 1993; Isacson *et al.* 2000). However, allograft surgery is subject to the same complications as autograft. Subsidence has been show to occur in cemented stems in 11% of 79 femoral prostheses in a study by Eldridge *et al.* (Eldridge *et al.* 1997). Masterson *et al.* also

reported subsidence using the Exeter impaction technique (Masterson *et al.* 1997). Other complications leading to failure are avascular necrosis, post-operative fractures (Heliotis and Tsiridis 2001), a high incidence of trochanteric non-union and prosthetic dislocation (Leopold *et al.* 2000). Subsidence suggests that graft is not sufficiently impacted or in the longer term that the graft has not incorporated into host bone (Heliotis and Tsiridis 2001). There is a possibility that problems associated with graft incorporation could be addressed by tissue engineering the graft, making it more osteoinductive.

1.3.4 Bone substitutes

It may be possible to engineer a graft that is more suitable for bone regeneration than allograft. This material must be porous and biocompatible i.e. non-toxic and non-immunogenic, enabling integration and bone ingrowth whilst at the same time being resorbable, thus allowing the graft material to withstand the forces imposed by everyday activity.

1.3.4.1 Synthetic HA

Synthetic HA is the most similar material to bone mineral compared to other synthetic ceramic substitutes. It is one of the most obvious choices as a bone substitute because it allows consistent reproduction, it is possible to manipulate its properties for use in various applications, for instance as an implant coating (Stephenson *et al.* 2003) or as blocks and granules of pre-specified sizes and shapes for grafting (Boyde *et al.* 1999; Oonishi *et al.* 2000) in combination with allograft or autograft (Oonishi 1991). It can mimick the porosity and pore sizes of the trabecular network, having interconnectivity for vascularisation and 200-900μm pores for cellular invasion. (Logeart-Avramoglou *et al.* 2004; Lanza *et al.* 2000; Salgado *et al.* 2004). HA coatings on implants led to osseointegration and the resultant increased rate of osseointegration increases mechanical stability faster than with a non-coated implant (Soballe *et al.* 1992). In another canine model, Stephenson *et al.* state that HA helped to close gaps at the bone-prosthesis junction (Stephenson *et al.* 2003). However, Buma *et al.* found that in a study of four retrieved HA coated implants, after a period of between 5-24 months, the HA had degraded leaving no bond between the implant and bone (Buma and Gardeniers 1995). HA is classed as a ceramic and is a relatively stiff material compared to

bone. It is also quite brittle and susceptible to failure because it is not reinforced by the presence of collagen. Disadvantages of HA as a graft substitute also include unpredictable dissolution rates *in vivo* (Salgado et al. 2004). This means that HA is not the perfect substitute for weight bearing bone grafts (Tachibana *et al.* 2003). These problems may be addressed in the future by utilising the knowledge of calcium phosphate chemistry to produce bioresponsive constructs that have bioactive molecules integrated with the substrate. As HA is resorbed these substances would be released and would act locally as osteoinductive factors. Currently HA cement is being developed to allow molding of the substrate into a specific defect (Gosain *et al.* 2003a).

1.3.4.2 Natural bone substitutes: Demineralised bone matrix (DBM)

Marshall R. Urist identified bone formation by autoinduction in 1965. He took samples of human and rabbit bone, decalcified in 0.6N Hydrochloric acid, and implanted them into the muscles of a variety of animals. The DBM was osteoinductive with new bone forming rapidly in weeks to months (Urist 2002). The mineral part of the matrix is thought to obscure the inductive properties of the matrix which are exposed by demineralisation (Lane and Sandhu 1987). Although this is good for craniofacial defects, the material properties of DBM mean that it is not suitable as allograft in weight bearing situations.

1.3.4.3 Growth factors

Growth factors, also known as cytokines, occur naturally in the bone matrix and act as signalling molecules between cells. They include the following: transforming growth factor β (TGF-β) superfamily containing bone morphogenic proteins (BMPs); insulin-like growth factors (IGF-1, IGF-2), involved in collagen synthesis and found in fracture healing sites; interleukins (IL-1, IL-6), involved in bone resorption; fibroblast growth factors (FGFs), involved in bone remodelling; platelet derived growth factors (PDGFs) involved in bone regeneration; and vascular endothelial growth factor (VEGF) which is angiogenic. Urist's discovery of autoinduction has been attributed to growth factors being exposed after demineralisation of the bone (Lane and Sandhu 1987) as it is known that BMPs are trapped within the bone matrix (Deans and Moseley 2000). It has been proposed that BMPs could be used to replace or augment autograft or allograft

administration in treating fracture non-union, accelerating the healing of bone defects and increasing union in distraction osteogenesis, the strength of bone union in spine arthrodesis and other arthrodeses and increasing bone mass in osteoporosis (Lane 2001). BMP-2 is thought to recruit and differentiate cells such as MSCs into the osteoblastic lineage (Deans and Moseley 2000) and recombinant BMP-2, 4 and 7 can heal critical sized defects in animal models when combined with a DBM, HA or biodegradable polymer carrier (Lane 2001). Clinical trials using BMPs have also been promising with clinical and radiological union in 82% of patients after BMP-7 application in persistent fracture non-unions and other orthopaedic complications (Giannoudis and Tzioupis 2005). The precedent was set in a clinical trial in tibial non-unions where patients either received intramedullary nail fixation and autologous bone grafting or the same procedure with the addition of recombinant BMP-7 (Friedlaender *et al.* 2001). Recovery was equivalent with both treatments and formed the basis of FDA approval for BMP-7 as an alternative to autograft in recalcitrant long bone non-unions under extenuating circumstances (Einhorn 2003). There was also a clinical trial using BMP-2 in lumbar spine disc degeneration where the fusion rate in the BMP treated group was higher than in the non BMP group (Einhorn 2003).

Despite the potential inherent in BMPs, the drawbacks are that animal models often show better bone healing than human clinical trials (Einhorn 2003). In mammals higher up the phylogenic scale, BMPs may need to persist in the vicinity of the carrier for longer (Lane 2001). This raises issues concerning systemic circulation of BMPs whereby a large dose may be toxic if not localised to the defect site. Very large pharmacological doses are used in relation to naturally occurring physiological quantities of BMP. In one study, concentrations of BMP extracted from the DBM of 20 patients were 21.4ng/g, 5.45ng/g and 84.1ng/g of BMP-2, BMP-4 and BMP-7 respectively (Pietrzak *et al.* 2006). However the effective quantity of BMP used in spinal fusions is about 1.5mg/ml of collagen carrier (Carlisle and Fischgrund 2005). In a randomised clinical trial, one unit of the rhOP-1 (recombinant human osteogenic protein-1) implant supplied by Stryker Biotech and used in the treatment of tibial non-unions contains 3.5mg of OP-1 per gram of collagen (Friedlaender *et al.* 2001). A maximum of two units was used and this by far exceeds the quantities found in human bone. Another concern is

that the action of BMPs may be dependent on stem cell recruitment, which may be compromised in a cohort of unhealthy patients compared to a healthy animal model. In addition, the application of large doses of BMP has been associated with osteoclast recruitment which may lead to a resorptive response (Lane 2001). There has been a recent study in sheep of impaction grafting with the addition of BMPs (McGee *et al.* 2004). New bone formation and graft remodelling was accelerated in the OP-1 group compared to the control. However, there was also an accelerated resorption at the early time-point in the OP-1 group which may compromise fixation of implants (McGee *et al.* 2004). The issues of toxity and controlled release of BMP may be addressed in the future with bioactive scaffolds that release bound growth factors in relation to the demands of the surrounding tissue. Apart from the pharmacological issues, there is the problem of expense and availability of commercially produced BMP (Einhorn 2003). For the purposes of this thesis I suggest that using cells alone with a scaffold will be self regulating, allowing the cells to produce the required amount of growth factors depending on the demands of the defect site.

1.4 Tissue engineering

1.4.1 Definition and history of tissue engineering

Tissue engineering usually involves the use of cells on a resorbable scaffold and sometimes biochemical cues to encourage appropriate cell function in a clinical application. A variety of scaffolds for bone tissue engineering have already been discussed. One of the main criteria for determining the scaffold is how the cells interact with the scaffold material *in vitro,* which will influence tissue regeneration *in vivo*. Tissue will only form through the activity of living cells (Muschler and Midura 2002). Tissue engineering as a commercial enterprise was initially pioneered by Organogenesis Inc. in the creation of skin grafts (Bell 2000). This cell based therapy was originally devised by Rheinwald and Green in the late 1970's using autologous keratinocytes to create epidermal grafts for burns victims (McPherson and Tubo 2000). Organogenesis' currently marketed product to treat venous leg ulcers and diabetic foot ulcers is Apligraf®, which is a cultured bilayer of allogenic dermis and epidermis, excluding cells of the immune system that initiate graft rejection (Organogenesis 2006). Another example of tissue

engineering in practice is autologous chondrocyte transplantation to treat cartilage defects of the knee. A biopsy of articular cartilage is extracted, enzymatically digested, the resulting chondrocytes cultured and reintroduced into an articular defect (McPherson and Tubo 2000).

A recent breakthrough towards applying stem cells to tissue engineering has been in skin grafting. Previously and unexpectedly, when human ES (embryonic stem) cells were purified they grew poorly. However. In a recent study, human ES derived keratinocytes were immortalised by transduction with viral genes and were maintained as keratinocytes in culture (Iuchi *et al.* 2006). There are several instances where stem cells have had a positive effect in treating a range of disorders. Peripheral blood stem cells have been used to treat leukaemia since the mid 1980's (Jansen *et al.* 2005). Also known as haematopoeitic stem cells (HSC), they are transplanted following irradiation of the patient. As irradiation is indiscriminate, killing healthy HSCs, leukophoresis is employed whereby donor HSCs are removed, expanded and transplanted back into the marrow after irradiation treatment (Jansen *et al.* 2005). In a recent study ES cells from the neural tissue of embryos have slowed the progress of Huntington's disease after implantation into the brain of 5 patients (Bachoud-Levi *et al.* 2006).

Examples of adult stem cells contributing to alleviation of disease in humans include: umbilical cord stem cells injected into the spinal cord of a 37 year old with spinal cord injury, regenerating the spinal cord, returning feeling and movement (Kang *et al.* 2005); allogenic corneal stem cells which were cultured on plastic and transplanted as a cell sheet onto the patient's corneal surface, resulting in improvements in the ocular surface and improved vision in 2 out of 4 people (Daya *et al.* 2005); and bone marrow derived autologous hemaotopoeitic stem cells injected directly into the myocardium resulted in a significant improvement in cardiac function when incorporated with coronary artery bypass grafting for congestive heart failure (Patel *et al.* 2005).

Mesenchymal Stromal Cells (MSCs) derived from bone marrow are considered most promising for bone tissue engineering. Bone marrow has been injected directly into tibial non-unions and showed a positive correlation between stem cell

concentration and volume of mineralised callus (Hernigou *et al.* 2005) and MSCs have also been used in a trial to treat osteogenesis imperfecta (Horwitz *et al.* 1999). These studies using bone derived stem cells did not employ scaffolds. **In my thesis, using MSCs in combination with a scaffold may solve some of the problems associated with allogenic bone grafts.** These advantages may be that:

1. It may redress the balance in favour of osteogenesis and away from osteoclastic resorbtion.
2. Graft may become more osteoinductive, increasing new bone formation.
3. Compared with autograft there is a greater supply of allograft.

1.4.2 Origin of MSCs and ES cells

The embryo consists of a mass of cells called the blastocyst, resulting in three germ layers that form all embryonic tissues: the ectoderm, endoderm and mesoderm. MSCs originate from the mesoderm and contribute to bone formation in the embryo, fracture repair and remodelling in the adult (Bruder *et al.* 1994; Shamblott *et al.* 2000; Shamblott *et al.* 1998). Blastocyst cells have the ability to regenerate an entire individual (Muschler and Midura 2002). The ability of these embryonic cells to give rise to the full complement of cell types can be defined as totipotent. Totipotent cells are the fertilised egg and the first 4 cells produced by its cleavage. Pluripotent cells have the ability to differentiate into any cell in the human body, as well as replicate themselves. There are three types of pluripotent stem cell: embryonal carcinoma (EC), isolated from teratocarcinomas; embryonic germ (EG) cells, isolated from the precursor to the gonads in aborted foetuses and ES cells, from the inner cell mass of the blastocyst (Shamblott *et al.* 2000). As the embryo develops into an adult, MSCs acquire a restricted potential to differentiate into skeletal tissue lineages such as: bone, cartilage, tendon, ligament, marrow stroma, fat, dermis, muscle and connective tissue, termed multipotent (Caplan 1991).

Whilst ethical considerations may prevent the use of ES cells in tissue engineering applications, adult MSCs may be obtained and cultured with less ethical concern (Orkin and Morrison 2002). ES cells have a tendency to form teratomas and teratocarcinomas *in vivo* (Salgado et al. 2004), so extra care has to be taken to differentiate the cells pre-implantation. Even with this precaution, there is still a

greater risk that unwanted tissue types will form *in vivo* when using ES cells compared with adult MSCs (Orkin and Morrison 2002). In order to maintain their undifferentiated status, human ES cells are cultured on murine matrigel or laminin in embryonic fibroblast conditioned media (Salgado *et al.* 2004). It is also academic that MSCs would be non-immunogenic as most uses would be autologous.

1.4.3 Definition of MSCs

MSCs can be defined as self-replicating cells that can give rise to multiple mesodermal tissues (Bruder *et al.* 1997). The definition of MSCs is under continual discussion and revision as researchers uncover new characteristics that suggest the boundaries of different cell types are shifting. The differences in nomenclature of the cell are generally semantic, with a variety of titles that have changed over time. However, the MSC is still generally characterised according to Friedenstein's *in vitro* observations of the phenotype in the 1970's (Friedenstein *et al.* 1970). After isolation from the bone marrow, MSCs selectively adhered to tissue culture plastic. This distinguished them from haematopoetic stromal cells (HSCs) of the marrow stroma which remained in suspension under tissue culture conditions (Hughes and Aubin 1998). Functionally, haematopoeitic cells are a population of stromal cells of the bone marrow distinct from MSCs that develop into mature blood cells (Young and Heath 2000). The adherent population of stromal cells were termed colony-forming unit fibroblasts (CFU-f) because in primary cultures they proliferate into colonies which are clones derived from a single cell (Friedenstein 1994). The term 'fibroblast' was allocated to these cells because their *in vitro* morphology exhibits a spindle shaped, fibroblastic appearance (Rickard *et al.* 1996; Friedenstein 1994; Owen and Friedenstein 1988). MSCs have a capacity for self-renewal, achieving more than 20 population doublings *in vitro* (Friedenstein 1994). *In vivo*, stem cells are thought to reside in specific niches that allow self renewal with descendants that can differentiate along a number of pathways (Watt and Hogan 2000). This results in a MSC repository that is most commonly associated with the marrow stroma. The advantage of this ability to replicate and maintain osteogenic capacity means that autologous MSCs can be sourced conveniently as a bone marrow aspirate, multiplied in culture and reintroduced at a defect site.

For the purposes of this thesis, the plastic-adherent cells derived from the bone marrow of the iliac crest will be termed mesenchymal stromal cells (MSCs). Where authors have used the acronym 'MSCs' to define 'Mesenchymal Stem Cells', it will be used with the knowledge that some authors may use the term interchangeably to represent 'Mesenchymal Stem Cells' and 'Mesenchymal Stromal Cells'. This is because the acronym 'MSC', as defined above, is a term that encompasses the origin of plastic-adherent cells derived from the bone marrow stroma without implying the differentiation potential or homogeneity of the population, which has not as yet been sufficiently characterised (Horwitz *et al.* 2005).

1.4.3.1 MSCs are multipotent and heterogenous

MSCs have been shown to differentiate into cells of the connective tissue lineage such as bone, cartilage, tendon, adipose tissue, fibrous tissue and myelosupportive stroma (Bianco and Robey 2000). The cells of these tissues are osteoblasts, chondrocytes, tenocytes, adipocytes and fibroblasts respectively. During MSC division, it is possible to retain the differentiation potential of the cells (Caplan 1991). In the bone marrow MSCs are only present in a ratio of 1:1,000,000 cells, (Hayensworth *et al.* 1992), but when cultured they continue to be multipotent and their number can be significantly increased. However, when Freidenstein transplanted single cfu-fs under the mouse renal capsule, they were shown to have different degrees of differentiation potential. Fifteen percent of colonies produced bone, adipose, marrow reticular tissue and host induced haemopoeisis forming heterotopic bone and marrow organ. Another 15% produced bone with osteocytes, but without an osteoblast layer or bone marrow cavity and the majority formed connective tissue without osteogenesis (Friedenstein 1980; Owen and Friedenstein 1988).

Confirmation of the plasticity of MSCs is exemplified in Freidenstein's *in vivo* assay. Rabbit marrow cells were loaded into diffusion chambers, implanted into the peritoneal cavity that excluded host cells but allowed nutrient diffusion. Histological evaluation confirmed cartilage development in the middle of the chamber and bone apposing the internal interface which was encased by

vasculature (Caplan 1991; Owen and Friedenstein 1988). These experiments also highlight the different environmental cues, triggering distinct phenotypic pathways. Cartilage formation requires a low nutrient and oxygen concentration which occurs in the absence of blood vessels. However bone formation requires high oxygen concentration, therefore a blood supply is required for osteogenesis (Caplan 1991).

1.4.3.2 MSCs are heterogenous

The reasons underlying heterogenity in MSC populations is a contentious issue. Caplan's experiment (Caplan 1991), described above, implies that the cells are multipotent because they are heterogenous. Heterogenity can be explained according to a number of theories. One theory is that asymmetric division can lead to a number of cell populations in different stages of the cell lineage, as supported by the work of Conget and Colter. Although MSCs often appear morphologically homogenous, they in fact display a heterogenity in their cell cycle *in vitro*. Analysis of DNA and RNA content by FACS (fluorescence activated cell sorting) after staining with acridine orange showed that 20% of MSCs at passage three were quiescent in the G_0 phase of the cell cycle and 70% were in the Gap 1 (G_1) phase (Conget and Minguell 1999). In the G_0 state, a cell exits the cell cycle reversibly or irreversibly and differentiates. The G_1 phase leads the cell into DNA synthesis and another cell cycle (Freshney 2000). According to Conget *et al.*, FACS analysis identified 3 populations of MSC that interact with each other. The small, a-granular, quiescent cells were termed recycling stem cells 1 (RS1) and were identified by staining with the Ki-67, an antibody directed against proteins in the cell cycle. RS-1 gave rise to RS-2 cells during the lag phase of growth, which in turn generate mature MSCs during the log phase. During the log phase, mature MSCs rapidly expanded. RS-2 cells declined with a concurrent increase in RS-1 cells during the late log phase (Colter *et al.* 2000). Essentially this means that the self-renewal of mature MSCs depends on the presence of RS-1 cells (Minguell *et al.* 2001).

A population of cells may show heterogenity due to the existence of committed progenitors already within the population. It is possible to derive multipotent cells from isolated individual colonies of human MSCs grown from a single cell.

Several experiments showed that these cells were differentiated into three different lineages commonly used to test multipotency: osteogenic, adipogenic and chondrogenic (Pittenger *et al.* 1999; Muraglia *et al.* 2000). Some clones showed restricted potential, which is partial evidence for the presence of committed progenitors with a more limited potential. *In vivo* these cells could be subject to sequential activation and some might just be genetically predisposed to be more self-renewing than others. Park's study shows a close association between the adipogenic and osteogeneic lineage with clones of adipocytes transdifferentiating with osteogenic lineage (Park *et al.* 1999). This reflects the inter-relationship of the cells with the *in vivo* marrow environment. For instance marrow is replaced by adipose tissue with aging (Prockop 1997). Heterogenity *in vivo* will certainly be influenced by so called 'niche' environmental cues such as diffusible factors (i.e. cytokines to be discussed later), extracellular matrix (ECM)-cell interactions and cell-cell interactions (Hall and Watt 1989). Some of these environmental cues are employed *in vitro* using physiologic concentrations of synthetic glucocorticoids such as dexamethasone and other supplements in order to indirectly characterise MSCs by differentiation (Jaiswal *et al.* 1997).

Another explanation for restricted potential of some clones is that culture conditions caused a loss in multipotentiality (Pittenger *et al.* 1999; Bonab *et al.* 2006). Characterising these cells would allow less potent cfu-f's in a heterogenic population to be discarded or set aside and it would be possible to pre-select a subset of cells that may already be slightly more committed to the bone lineage. The osteogenic potential of marrow varies from patient to patient (Phinney *et al.* 1999), and decreases with age in women (Muschler *et al.* 2001) so expansion of MSCs *in vitro* would allow bone remodelling in patients with a low MSC cell count, i.e. patients with age related disorders like osteoporosis (Bruder and Fox 1999). Osteogenic commitment and differentiation *in vitro* prior to implantation may provide more rapid and consistent bone formation (Caplan A.I., 2000). On the other hand, it may be beneficial to transplant a heterogenous population of cells that would already be interacting via cell-cell communication in order to adapt better to the *in vivo* environment. **This is why, in my thesis, I will be using both pre-differentiated osteoblasts from MSCs and non differentiated MSCs.**

1.4.4 Tissue source of MSCs

MSCs have been found to maintain a reservoir in many adult tissues of the body. MSCs have been isolated from several human, skeletally derived tissues including: marrow (Rickard *et al.* 1996), periosteum (Caplan 1991; Hutmacher and Sittinger 2003), muscle (Caplan 1991) and fat (Gronthos *et al.* 2001; Park *et al.* 1999). Isolation of these cells has also been successful from a variety of animal tissues including fetal calvaria, chick limb buds (Caplan 1991) and rat heart muscle (Warejcka *et al.* 1996). The marrow stroma is thought to be the primary reservoir for these self replicating cells (Beresford 1989; Owen 1985). The word stroma literally means 'matress: anything spread or laid out for lying upon' and this is exactly what the stroma of the bone constitutes (Owen 1985). Stroma is connective tissue within the marrow that provides structural and functional support for haematopoesis (Beresford 1989). By definition this suggests that stromal cells are interconnected and unmoving, supporting the mobile cells of the haematopoetic system, which is the other main cell compartment of the bone marrow (Kuznetsov *et al.* 2001).

However, there is some evidence of MSCs circulating in the peripheral blood (Kuznetsov *et al.* 2001) and umbilical cord blood (Erices *et al.* 2000), which challenges previously held opinions that such cells are quiescent and non-circulating (Bianco and Robey 2000; Kuznetsov *et al.* 2001). The rationale behind this is that HSCs are required to replicate continually to maintain the number of cells at a constant level (Hall and Watt 1989) whereas MSCs embed in the tissue, respond to injury or remodelling, which leads to their differentiation and limits their self renewing potential (Hall and Watt 1989; Bianco and Robey 2000). Evidence for this is that self-renewal is lost as commitment to a lineage increases (Colter *et al.* 2000). However MSCs are still purported to be found in the blood system, suggesting that they can be mobilised in response to injury and have an inherent homing capability (Minguell *et al.* 2001; Devine *et al.* 2003). The homing capacity of MSCs and subsequent regeneration of damaged tissue is supported by research on skeletal muscle and the heart. In one paper lacZ marked mouse MSCs were injected into irradiated mice that had chemically induced muscle regeneration by injection of cardiotoxin in the tibialis anterior (TA). MSC

derived myogenic progenitors were detected in both immature and mature muscle fibres in the TA by β-galactosidase staining (Ferrari *et al.* 1998). Barbash, I.M. *et al.* demonstrated that intravenous infusion of MSCs in rats with myocardial infarction led to MSC engraftment in the damaged tissue of the heart in preference to intact or remote myocardium, although direct left ventricular cavity infusion led to significantly greater engraftment in the ischemic heart (Barbash *et al.* 2003). Furthermore, when allogenic MSCs were injected into the jugular vein of MI (myocardial infarction) induced pigs and analysed for parameters of heart function after 3 months, they were found to have less hypertrophy and left ventricular ejection fraction than control animals. In addition small numbers of MSCs were found in injured portions of the heart (Price *et al.* 2005). These discoveries increase the possibilities for therapeutic intervention using stem cells. If a systemic injection of cells could be used it may be an expedient form of transfusion similar to the principles of leukophoresis.

1.4.5 Isolation and characterisation of MSCs

Characterisation methods *in vitro* have primarily relied on differentiation of an adherent population of cfu-fs into the three different lineages of skeletal tissue cells: osteoblasts, chondrocytes and adipocytes (Pittenger *et al.* 1999). It is possible to identify these cells by morphology; cell surface characteristics, such as antigens for immunophenotyping; and synthesis of extracellular matrix and biochemical molecules. Markers for adipocytes include intracellular lipid vacuoles, the presence of peroxisome proliferator–activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL). Chondrocytes will present collagen X and collagen II (Pittenger *et al.* 1999). The production of osteoblasts and bone is associated with a number of organic components of bone synthesised by the cells at different stages of the lineage progression. Some examples of the extracellular matrix proteins and enzymes produced by osteoprogenitors and the mature osteoblast phenotype that are used commonly for identification *in vitro* include: collagen I (Gronthos *et al.* 2001), OC (Muraglia *et al.* 2000), alkaline phosphatase (ALP) (Jaiswal *et al.* 1997) and OP (Gronthos *et al.* 1994). Collagen has previously been discussed as the most abundant protein in the organic matrix of bone. It is secreted by osteoblasts into the extracellular matrix and is composed of a triple helix of tropocollagen molecules. The tropocollagen aggregates into fibrils which are congregated into

bundles (Young and Heath 2000). Collagen I confers tensile strength to the tissue and is secreted throughout osteoblast differentiation (Hughes and Aubin 1998). However, collagen I is produced by a number of other cell types and is not specific to osteoblasts. ALP is an enzyme that has been described as prompting phosphate ions to accumulate on collagen fibrils. Osteoblasts express the tissue non-specific form of the enzyme which is highly expressed in bone, liver and kidney (Young and Heath 2000). It is expressed in differentiating osteoblasts *in vitro*. It makes sense that ALP peaks in expression at day 12 and then declines (Bruder *et al.* 1997) because the function of ALP is to encourage mineral deposition which requires collagen to be present first. OC is the most abundant non-collagenous protein in bone and may have a role in bone remodelling (Beresford *et al.* 1984). OC production is almost exclusive to osteoblasts (Hughes and Aubin 1998). OP may have a role in attachment of cells to bone matrix via the Arg-Gly-Asp sequence of amino acids (RGD). Less commonly used as markers are ON, and proteoglycans, such as decorin and biglycan, because they are either expressed at low levels or are expressed by other cell types within the bone matrix (Hughes and Aubin 1998). As the expression of proteins within each cell type can overlap (i.e. ALP is expressed in many cell types and OP is expressed in osteogenic and chondrogenic pathways) (Pittenger *et al.* 1999), the most reliable identification of osteogenic differentiation is a gamut of protein assays or a mineralisation assay. The transcription factor cbfa-1/Runx2 was found to induce and regulate expression of multiple genes in osteoblasts and chondrocytes (Karsenty *et al.* 1999; Ducy *et al.* 1997) and may be used as a specific marker to identify the preosteoblast phenotype (Nakashima *et al.* 2002). In addition, the transcription factor Osterix acts downstream of Cbfa1/Runx2 to induce differentiation of preosteoblasts to osteoblasts (Nakashima *et al.* 2002).

Each protein can be described within the context of its physiological role in bone formation and remodelling, or the temporal role it plays in osteoblast differentiation *in vitro* where investigators have often seen different patterns of expression with respective proteins. There is a changing pattern of expression of each marker during osteoblastic differentiation. Investigators have observed different patterns of expression between species and different cell lines. Variation

could also be due to the heterogenity of the population of MSCs under investigation (Hughes and Aubin 1998).

Human MSCs have been investigated more thoroughly than any other species. There has still not been a single specific marker that completely identifies the multipotent MSC (Baksh *et al.* 2004). It is a common consensus that mesenchymal stromal cells do not express heamatopoeitic antigens CD14, CD34 and CD45 (Pittenger *et al.* 1999; Conget and Minguell 1999). The immunophenotype of the MSC is defined by the antigens it lacks when compared to haematopoetic cells, rather than specific antigens on its surface. This may be a problem as antigens on MSCs tend to be present on cells of other tissue types. The antibodies SH2, SH3 and SH4 were found to react with MSCs and not haematopoietic cells, osteoblasts or osteocytes. However, they do cross react with fibroblastic cells (Hayensworth *et al.* 1992). The STRO-1 antibody is similar in that it identifies early osteoblast progenitors, but is not expressed exclusively by the MSC (Simmons and Torok-Strob 1991). The expression of the antigen detected by STRO-1 diminishes as cells begin to express the mature osteoblast phenotype (Gronthos *et al.* 1994). So STRO-1 is another marker that is dependent on the lineage progression of the MSC (Wülling *et al.* 2003). Figure 1.4 shows the antibodies that can be used to characterise each cell of the osteoblastic development lineage in humans (Wülling *et al.* 2003). The cell surface markers for human MSCs are shown in the table below (Table 1).

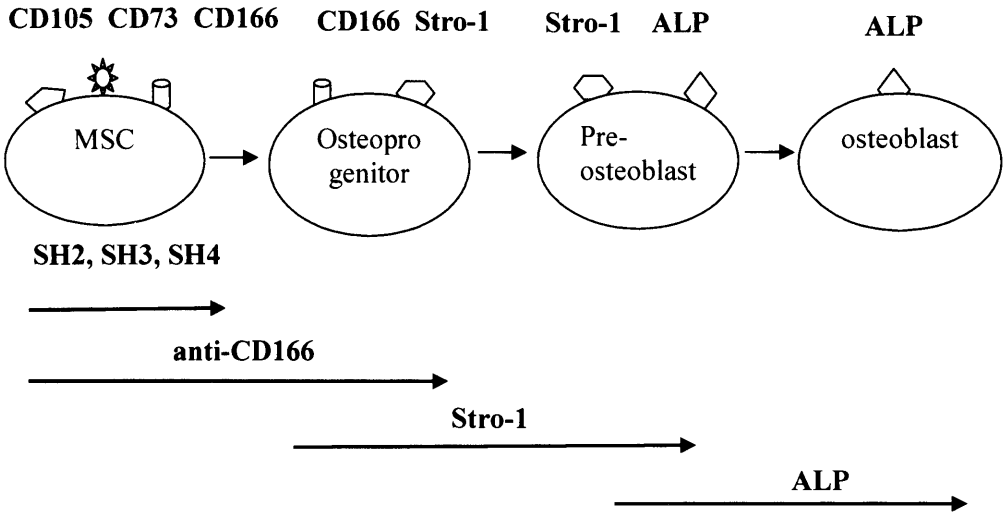


Figure 1.4 Osteoblastic differentiation and marker expression (Wülling *et al.* 2003).

Phenotypic characterisation of multicolony derived strains of MSCs		
Marker expression	**Human MSCs (Kuznetsov et al., 2001)**	**Human MSCs (Deans and Moseley, 2000)**
Fibroblastic		
Col I	++	
Col III	++	
CD44	++	
ß1 subunit	++	
Osteogenic		
ON	++	
OPN	-+	
BSP	-+	
OC	-+	
Adipogenic		
CEBPα1	-+	
PPARγ2	-	
Endothelial		
CD34	-	Neg
Muc-18 CD146	+	Pos
FactorVIII	-	
Endoglin	+	
Marrow stromal cells		
Stro-1	-+	
CD106 (VCAM-1) Adhesion molecule	+	Pos
Smooth muscle		
α-smooth muscle actin	-+++	
Skeletal muscle		
MyoD	-	
Monocyte/Macrophage		
CD14 Dako LPS receptor	-	Neg
Leukocyte		
CD45 (Dako) leukocyte common antigen	-	
Nerve		
Neurofilament (Dako)	-	
Histochemical		
ALP	-+++	
Acid Phosphatase	+	
A-Napthyl acetate esterase	+	
Oil red O		
20% FBS	-+	
20% rabbit serum	++	
Adhesion molecules		
ALCAM CD166		Pos
ICAM-1 CD54		Pos
ICAM-2 CD102		Pos
E-selectin CD62E		Neg
L-Selectin CD62L		Pos
P-selectin CD62P		Neg
LFA-3 CD58		Pos
Cadherin 5 CD144		Neg
PECAM-1 CD31		Neg
NCAM CD56		Pos
HCAM CD44	++	Pos
Hyaluronate receptor		Pos
Growth factors & cytokine receptors		
IL-R (α OR β) CD121a,b		Pos
IL-2r CD25		Neg
IL-3R CD123		Pos
IL-4R CD124		Pos
IL-6R CD126		Pos
IL-7R CD127		Pos
Interferon γ R CDw119		Pos
TNF- α-IR CD120a		Pos

Table 1.1 Cell surface markers for human MSC

1.4.6 Application of MSCs with 3D scaffolds *in vitro* and *in vivo*

There are two approaches to tissue engineering. One is to attempt to grow tissues *in vitro* with a view to implanting the entire regenerated organ in place of the damaged one. The other option is to partially grow the tissue *in vitro* in order to allow for regeneration of the organ *in vivo*. Most attempts at regeneration of bone tend towards the latter. The combination of HA, or constructs incorporating HA, with MSCs or MSC derived osteogenic cells, has proved feasable as a tissue engineered scaffold for ectopic bone formation in small animal models such as mice (Gundle *et al.* 1995; Harris and Cooper 2004; Gundle *et al.* 1997) and rats (Okumura *et al.* 1996). The diffusion chamber is a system that can be used to isolate either autogenic or allogenic MSCs which allows the effects of stem cell origin to be measured. In these studies the nature of the enclosed system means that bone that is produced is derived soley from cells within the diffusion chamber (Gundle *et al.* 1995; Gundle *et al.* 1997), as diffusion chamber membranes are not permeable to cells. When MSCs were loaded onto various HA containing matrices, cells induced bone on 2 out of 4 constructs after implantation into the dorsum of severe combined immunodeficient (SCID) mice compared to controls. This showed that MSCs were required for osteogenesis. However some matrices were not inducible, despite the introduction of cells (Harris and Cooper 2004).

Ectopic models have also been applied in large animal models to elucidate the osseoinductivity of MSCs. All the tissue engineered constructs containing HA and MSCs formed bone when implanted into the paraspinal muscles of goats (Kruyt *et al.* 2004b). Orthotopic, segmental defect models are more representative of the surrounding bone tissue and loading regimens. MSC loaded HA constructs were implanted into segmental defects in rat femora. They achieved superior union of the host-implant interface compared to marrow loaded or cell free implants. However, the diffusion chamber test and ectopic models do not accurately reflect the loading demands on orthopaedic implants (Lane and Sandhu 1987). Additionally, the results on bone regeneration from segmental defects in animal models cannot necessarily be translated into the use of tissue engineering

constructs for impaction grafting. A large animal study in goats showed that all constructs required the addition of MSCs to induce bone formation as, after implantation in the paraspinalis muscles, there was no sign of bone formation in control constructs alone (Kruyt *et al.* 2003). Conversely, another study by the same group comparing the results of MSCs on HA at ectopic and orthotopic locations suggests that cells make a limited contribution to constructs in an orthotopic location. It was observed that there was significantly more bone apposition at the shortest timepoint compared with larger bone contributions at early and late timepoints in the ectopic model (Kruyt *et al.* 2004c). Successful tissue engineering to repair segmental defects in large animals include: the use of allogenic (Arinzeh *et al.* 2003) and autologous (Bruder *et al.* 1998b) MSCs seeded onto HA cylinders to regenerate femoral, canine segmental defects; MSCs seeded onto HA cylinders implanted into sheep tibial segmental defects, where bone formed more extensively and indentation testing revealed greater stiffness in cell loaded constructs (Kon *et al.* 2000), and similar results were found in the work of Chistolini *et al.* (Chistolini *et al.* 1999). The clinical union of critical size defects in sheep metatarsals on tissue engineered coral cylinders with MSCs was defined by continuity of both cortices on radiographic analysis (Petite *et al.* 2000). Since metatarsal diaphyses contain no trabecular bone and have different loading regimens, it is difficult to compare with femoral or tibial defect models. However, there was an increase in clinical union in MSC treated samples compared to marrow or coral alone and some evidence of osteogenesis in the medullary areas in MSC loaded samples. Blokhuis's group used HA particles as a carrier for autologous marrow in an ovine segmental defect model. The results show that the addition of bone marrow did not lead to acceleration of bone healing compared to autologous bone grafting (Blokhuis *et al.* 1999). However this may be a factor that is associated with the use of dense HA granules rather than MSCs.

1.4.7 Clinical application of tissue engineering using MSCs

The mobilisation and transplantation of HSCs has been an accepted form of cancer treatment in patients undergoing chemotherapy since the 1990's. Leukaphoresis can be applied as an autogenic or allogenic treatment whereby mobilisation or transplantation reconstitutes hematopoeisis repectively. The application of the

growth factors Granulocyte colony stimulating factor (G-CSF) and Granulocyte-macrophage colony stimulating factor (GM-CSF) reconstitutes white blood cells and also boosts hematopoeisis, making the process more efficient (Jansen *et al.* 2005). While use of haematopoetic cells is commonly accepted, the clinical transplantation of MSCs has just begun and is in the early stages of investigation. A clinical study of three patients with osteogenesis imperfecta indicates that allogenic bone marrow-derived MSCs transplanted by intravenous infusion can migrate to bone, improving bone structure and function (Horwitz *et al.* 1999). Autologous transplantation of bone marrow has been attempted in tibial shaft non-unions. Although there was no negative control, increased callus formation was associated with the number and concentration of cfu-fs in the marrow (Hernigou *et al.* 2005). However there have been no investigations into the use of MSCs to regenerate new bone in a revision total hip replacement.

Aim

To enhance new bone formation around revision THRs by incorporating mesenchymal stromal cells (MSCs) with allograft used for impaction grafting.

Hypothesis

Incorporating MSCs into allograft or HA granules which are then impacted, will increase the rate and extent of bone formation.

CHAPTER 2: *In vitro* Impaction of Mesenchymal Stromal Cells on an Allograft Scaffold

2.1 INTRODUCTION

One of the effective methods currently used to regenerate bone stock at revision surgery is impaction of cancellous bone graft with or without bone cement. This technique has been shown to provide structural and functional support encouraging ingrowth of host bone (Tägil 2000; Soballe *et al.* 1992). Impaction occurred with cement was first used in combination with implants to treat acetabular protrusion (Sloof *et al.* 1984). This technique was then developed by the Exeter Group for fixation of the revison femoral component (Gie *et al.* 1992; Gie *et al.* 1993). Impaction occurred with cement does have considerable advantages and to date, results from various centres with follow-ups ranging from 2-10 years have re-revison rates for aseptic loosening of between 4 and 8 % (Meding *et al.* 1997; Leopold *et al.* 1999; Mikhail *et al.* 1999). Histology from biopsy samples has shown viable tissue growth into the graft (Ling *et al.* 1993). However, regeneration of new bone within defects surrounding revision implants has shown rather limited and inconsistent results (Ullmark and Obrant 2002; Mikhail *et al.* 1999).

A number of animal studies have demonstrated that tissue engineering techniques using autologous mesenchymal stromal cells (MSCs) combined with a suitable carrier, can create new bone in the defect site (Bruder *et al.* 1998b; Kruyt *et al.* 2003; Kon *et al.* 2000). A combination of tissue engineering techniques using autologous MSCs with clinical impaction of allograft in revision surgery may be able to regenerate bone stock and improve long-term implant fixation. However, one question that has to be answered is: Can MSCs seeded onto allograft survive after undergoing normal impaction forces used for revision THRs?

Aim

To answer this question, a study was carried out *in vitro* to test the viability of the MSCs which were seeded onto the allograft and impacted at a range of forces obtained during revision THRs.

Hypothesis

MSCs seeded onto allograft scaffolds will survive normal impaction forces.

2.2 MATERIALS AND METHODS

2.2.1 Harvesting MSCs

2.2.1.1 Obtaining bone marrow

Bone marrow from the iliac crest was aspirated from nine sheep. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 at the Royal Vetinary College, North Mymms. Home Office Licences were held by all those taking part in any surgical procedure.

Sheep were premedicated with intramuscular Xylazine at 0.1mg/kg 10 minutes pre-operatively. Induction of anaesthesia was by intravenous injection of Ketamine 2mg/kg and Midazolam 2.5mg. The animal was then intubated and maintained on 2% Halothane and oxygen for the duration of the aspiration. Intraoperative monitoring consisted of pulse oximetry, ECG and end tidal carbon dioxide.

The sheep were placed in the left lateral position and the fleece above the right iliac crest was shaved to the skin. Pevidine surgical scrub was used to wet the area at 1 part pevidine to 9 parts water. Neat pevidine surgical scrub was then applied and worked into a lather. This was left for two minutes. The area was further sterilised with pevidine antiseptic solution by swabbing the area with a spiral motion from the □steoc of the aspirate area outwards. A swab was used to wipe down the entire area with Hydrex solution-chlorohexidine and the whole area was covered with a drape.

A stab incision was made down to the iliac crest under sterile conditions. The bone marrow gauge needle was twisted into the intramedullary cavity. A 10ml syringe pre-loaded with 1ml heparin at 1000 iu/ml was attached to the marrow gauge needle and used to aspirate 3ml of bone marrow from the intramedullary cavity of the iliac crest. The marrow was then transferred into a sterile universal tube, swirled slightly to mix the heparin with marrow to prevent clotting and placed on ice for transfer to tissue culture facilities. A subsequent two aspirates were taken from different positions, working from anterior to posterior, on the iliac

crest in the same manner as described above. After the procedure was completed the incision was sutured using a resorbable zero vicryl™ suture.

2.2.1.2 MSC Isolation

The bone marrow and heparin mixture, containing a heterogeneous population of cells, was centrifuged down a Ficoll® (Amersham Pharmsia Biotech, UK, 17144002) sugar-sucrose gradient in order to isolate a homogenous population of mononuclear cells consisting of MSCs. MSCs were further selected by attachment to tissue culture plastic *in vitro*. Firstly, 3ml of Ficoll® was added to a universal. The aspirate was gently dispensed down the side of a universal onto the Ficoll® to give a bottom layer of Ficoll® and an upper layer of bone marrow. The sample was centrifuged at 1510 rpm (400g) for 30 minutes, leaving a straw coloured buffy layer at the interface between a ficoll® erythrocyte residue at the bottom and a plasma layer above. A transfer pipette was used to remove the buffy layer, containing mononuclear cells, to a separate universal.

The buffy layer was resuspended in 10ml Dulbecco's modified eagles medium (DMEM, Sigma D6429), spun at 1500rpm for 10 minutes to pellet the mononuclear cells, leaving the Ficoll® and heparin in suspension which was subsequently discarded. The cell pellet was resuspended through a 23 gauge needle using 2ml of DMEM to give a single cell suspension. The pellet was resuspended in 10ml DMEM, centrifuged at 1500 rpm for 5 minutes. The pellet of mononuclear cells was further washed by resuspending and centrifuging before being finally resuspended in DMEM and plated into two 25cm^2 flasks. Cells were maintained in incubators at 37°C with 5% CO_2 in growth media consisting of Dulbecco's modified eagles medium (DMEM) supplemented with 10% FCS (First Link, West Midlands, UK), penicillin / streptomycin (1000iu/ml; 100µg/ml) (GIBCO Paisley, UK). These conditions remained the same for all *in vitro* cell culture procedures unless stated otherwise.

2.2.1.3 Cell culture

A defining characteristic of MSCs that enables their selection *in vitro* is their adherence to tissue culture plastic (Colter *et al.* 2000; Friedenstein *et al.* 1976;

Rickard *et al.* 1996; Okumura *et al.* 1996). Primary seeded cells were allowed to adhere to tissue culture plastic for two days before the DMEM was replaced, thereby diluting and eventually removing any haematopoietic cells from the suspension. DMEM was replaced every two to three days and cells were grown to subconfluence before harvesting. Subconfluence is a state of growth where not all the available flask surface is covered. The limit of subconfluence was defined by eye at 60-90% coverage. Confluence is where total growth area is used and MSCs make close contact with one another. It was important that cells did not reach confluence at any point during culture. This is because MSCs will maintain a consistent phenotype if the cell density is kept low and be less susceptible to spontaneous transformations (Freshney 2000). Transformation is a result of genetic instability where spontaneous or induced permenant phenotypic change results from a heritable change in DNA and gene expression: immortalisation, aberrant growth control and malignancy (Freshney 2000).

Expansion of the cells was continued by trypsinisation, to remove the cells from the flask surface, washing and re-plating into new flasks. DMEM was removed from the flasks. The flask was rinsed by the addition of 5ml PBS. Trypsin (Sigma T8003) was then added at a volume of 2.5ml and a concentration of 10% with 1% HEPES buffer. The flasks were then placed in an incubator for 5 minutes to allow the enzyme to release cells from the tissue culture plastic. The flasks were agitated to further release cells. Enzyme activity was quenched with the addition of 5ml DMEM. All cells were removed to a universal and centrifuged at 1500rpm to pellet the cells and remove trypsin into suspension. Trypsin was removed and cells were resuspended in 10ml DMEM to further wash the cells. After the final centrifugation at 1500rpm, cells were resuspended in DMEM and replated at passage 1 (P1) into three T75 flasks. Cells were trypsinised into larger flasks on subsequent passages. Cells in this study were used between passages 3 and 6. This cell culture technique was used for all *in vitro* cell culture unless stated otherwise. Cells were counted after resuspension in 1ml of DMEM. An equal volume of this suspension and trypan blue were mixed together and the cells were introduced into a coverslipped haemocytometer. Cells stained blue were considered dead whereas viable, bright and rounded cells were counted where they

fell within the bounds of haemocytometer divisions. This procedure was used for all subsequent cell counting.

2.2.1.4 Growth curve for sheep MSCs in monolayer

In order to ensure that cells were impacted at the most proliferative and durable stage of development, a growth curve was constructed. A series of cultures were set up at a cell density of 3 x 10^4 cells/ml and the cells were counted daily until they reached the plateau phase. Three cell lines (n=3 animals) were cultured to passage 4 using standard tissue culture techniques previously described. The MSCs were trypsinised into suspension with 3 x 10^4 cells/ml in 25 ml of medium. The cells were seeded onto 24 well plates with 1ml of cell suspension in each well. The cultures were maintained at 37°C with 5% CO_2 in the original growth media and cells were counted from three wells of each plate every 24 hours for five days.

2.2.2 Processing long bones for allograft

2.2.2.1 Harvesting ovine long bones

Allograft was made following North London Tissue Bank Protocols. Fresh ovine femoral bones were harvested from the Royal Veterinary College, North Mymms. Femoral bones were frozen immediately at -20°C until retrieval for processing. Bones were thawed for 30 minutes and any attached soft tissue and cartilage were removed. Cortical bone was removed by trimming on the Draper bandsaw until only trabecular bone remained, which was further cut into manageable pieces.

2.2.2.2. Morsellising, defatting and irradiation of graft

Sections of bone were passed through the Lere Bone Mill to morsellise the graft. Further processing by washing the morsellised graft removed protein, cells and tissue, reducing the possibility of an immune response of the recipient to donor antigens, disease transmission or extraneous contamination. The water in all the washing steps was maintained at 55°C. The morsellised graft was washed by placing in an ultrasound bath filled with water and sonic washed for 15 minutes to remove cells and blood. The following series of washes were: three consecutive water washes of 15 minutes, a water wash for 60 minutes, a 10 minute wash with

70% alcohol to denature cellular proteins and kill viruses and bacteria, followed by two final water washes. The allograft was patted dry ready for sieving. All the graft was placed at the top of the pile of sieves and shaken for half an hour until all the graft had fallen into its relevant sized sieve, so that graft was sieved to a uniform size. For this study, the graft size was in a range of 2.4-4.76mm. The allograft was sent to Isotron on dry ice to be gamma irradiated at 25Kgrays (Isotron, Reading, UK). Allograft was maintained in storage at -20°C.

2.2.3 Determining impaction forces

2.2.3.1 Modified impactor

The impaction data used in this project was based on the Exeter X Change system and was previously collated by Kirsty Phipps at the Centre of Biomedical Engineering (Phipps *et al.* 2002). In order to establish impaction forces used in revision THR, the Exeter Slap Hammer was modified to accommodate a piezo electric load washer (figure 2.1). Force readings were emitted by the load washer at each impaction which were converted into voltage and recorded on a specialized computer program (Skylark Ltd, London, UK). By impacting graft enclosed within a sawbone, using a proximal impactor fitted with a load washer using the instrumented hammer also containing a load washer, it was possible to measure the forces imparted to the graft material and relate these to the measured hammer forces.

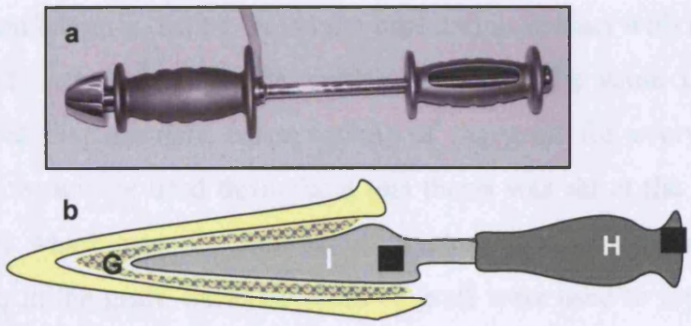


Figure 2.1a The Exeter slap hammer; **b** Hammer forces measured in the graft (G) impacted in a sawbone using load washers (denoted by black squares) in hammer (H), and in proximal impactor (I) (Phipps 2004)

2.2.3.2 Drop heights to replicate forces used in a normal surgery

The normal range of impaction forces measured by the modified Exeter impactor was reproduced in an *in vitro* impactor. The piezo electric load washer was

attached to the impactor to record forces in different height of drops. 3.5g of allograft was added to a Delrin tube which was 15mm in diameter and 40mm in height. The 1kg weight on the impactor was dropped onto allograft contained within the tube (figure 2.2). Impactions were performed at a range of heights from 2.5 to 20cm with increments of 2.5cm. Readings were taken 10 times at each height. Graft was changed after each height and force readings were recorded. A calibration graph of force versus drop height was constructed.

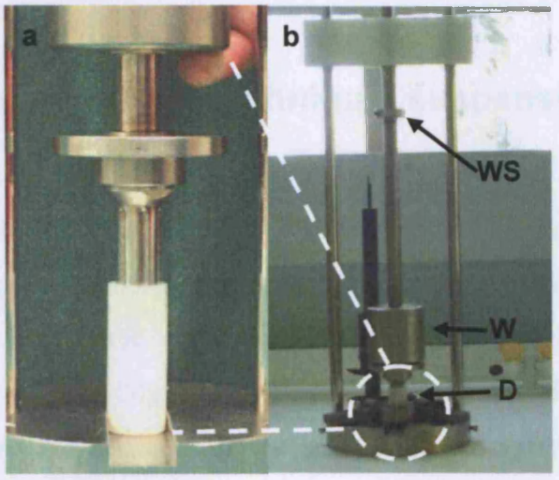


Figure 2.2a Impactor; **b** Detail of impactor with Weight (W), Weight stop (WS) and Delrin tube (D)

2.2.3.3 Optimum number of impact cycles to achieve maximum compaction

The graft material was contained within a Delrin tube. A punch directly attached to the impaction hammer, fitting inside the tube and in contact with the graft, delivered the force of each impact to the graft at 3kN. At the same time a micrometer measured the displacement (compaction) of the graft for every impaction. The number of impactions used throughout this thesis was set at the limit of maximum compaction, which was the number of cycles after which there is little or no deformation in the graft. Different sizes of graft were used to represent the choices made in surgery. Small graft was between 2.0-3.2mm and the large graft consisted of granules in the size range 4.76-6.68mm. The graded graft was a mixture of graft sizes (Phipps 2004).

2.2.3.4 The collapse of graft under different loads

Samples of 3.5g of allograft were placed into 3 Delrin tubes. Each tube was impacted at 3, 6 or 9kN. Readings were taken 100 times at each force to measure the degree of collapse of the graft. A graph was plotted to determine whether a larger number of impactions at a lower force could achieve the same degree of compression of the graft as a smaller number of impactions at a higher force. Using less force to achieve the same degree of impaction may maintain the viability of the cells seeded onto the graft.

2.2.4 Determining seeding technique: suspension or density seeding

Sheep MSCs were trypsinised from tissue culture flasks, all cells were subconfluent at passage 3. Sheep MSCs were seeded at 2 x 10^6 cells/g onto 3.5g of allograft in a universal tube with 7ml DMEM 10% FCS and penicillin / streptomycin (1000 iu/ml; 100µg/ml). Another sample of 3.5g of allograft was placed in a petri dish and seeded with 2x10^6 cells/g by density seeding. Density seeding was achieved by dispensing the cells suspended in 500µl of DMEM onto each individual piece of allograft using a Gilsons pipette. The cells were left to adhere to the graft for 30 minutes in the 37°C incubator, at which point the graft was flooded with 7ml DMEM. There was one allograft sample with no MSCs which was the control. After 72 hours under tissue culture conditions, two granules from each sample were processed for SEM analysis. The seeding technique was chosen based on even coverage of the graft by the MSCs.

2.2.5 *In vitro* impaction of MSCs on allograft

2.2.5.1 Suspension seeding of MSCs onto allograft for impaction

The decision to suspension seed MSCs onto allograft in a rotator was due to the findings from SEM analysis. MSCs were trypsinised from culture flasks, counted and seeded at 2 x 10^6 cells/g onto 3.5g of allograft in a universal tube with 7ml DMEM, 10% FCS and penicillin / streptomycin (1000 iu/ml; 100µg/ml). All cells were sub-confluent at passage 3. Control universal tubes (n=9) contained allograft only with 7ml of DMEM. Cells were attached to the allograft by rotation on a TAAB rotator (TAAB, UK) spinning at 4rpm for 2.5 hours at 37°C with 5% CO_2.

The cells were then transferred to a 6 well plate and were maintained in culture for 72 hours pre-impaction, as determined by the growth curve. There were 9 samples for each force group, including the 0kN control where MSCs were added but no impaction force was used. In addition, two granules of graft were removed from each sample for SEM analysis after 2.5 hours and 72 hours in culture.

2.2.5.2 Impacting constructs in Delrin tubes

Allograft at 3.5g was added to a Delrin tube which was 15mm in diameter and 40mm in height. Impaction forces of 3kN, 6kN and 9kN were selected because they represented low, medium and high impaction forces measured by the instrumented slap hammer during surgery. The forces were regulated by the drop height of the 1kg weight attached to the impactor which was specially designed for this study (Figure 2.2a & 2.2b). 20 impactions were applied at each selected force. 0kN was used for the control group.

2.2.5.3 Alamar Blue™ Assay

The graft conduits were analysed using a 1 in 10 dilution of Alamar Blue™ (Alamar Biosciences, UK) with phenol red free DMEM, after washing in PBS. A solution of Alamar Blue™ is a biochemical indicator of cells metabolism that changes from blue to pink in response to redox reactions in the cytochrome oxidase chain. Samples were incubated in Alamar Blue™ for 4 hours at 37°C, 5% $C0_2$. The Alamar Blue™ solution was then removed and absorbance was read at 570nm with a reference wavelength of 630nm on a Fluoroscan Ascent plate reader (Labsystems Inc, USA). The grafts were then assayed with a 1 in 10 dilution of Alamar Blue™ after samples were impacted. For continued culture, allograft conduits were washed twice with PBS and fresh DMEM was added to each well. The samples were then assayed in the same manner daily for six days and maintained in culture at 37°C with 5% CO_2 with a change of culture medium every day. One granule of graft was taken from each impaction group after six days for SEM analysis. One sample from each group was fixed for resin sectioning after six days.

2.2.5.4 Resin embedding of samples

To demonstrate the presence and distribution of MSCs on allograft samples, after impaction and incubation for 6 days, one sample from each impaction group was processed in LR White hard grade resin for analysis (as above). Initially samples were rinsed for five minutes in PBS. This was repeated 3 times then samples were fixed with neutral buffered formalin for one day. Samples were placed back into cylindrical Delrin tubes. These were then placed into containers and covered with 50% industrial methylated spirits (IMS) in distilled water for one day. The solution was discarded and 100% IMS was added and left for one day. Following this, 50% LR White hard grade resin (Agar) in IMS was added to the sample which was placed under vacuum to ensure complete penetration of the graft with resin. The vacuum was replenished every day for two days. Finally this solution was discarded and 100% resin was added to the samples and subjected to a vacuum every day for three days. The samples were cast in resin. All solutions were discarded, and one drop of catalyst was added for every 10ml of resin and mixed using a magnetic stirrer. This was poured onto the sample. The sample was placed in the fridge so that the resin would set slowly at 4°C. The samples were sectioned in the transverse plain using the Exakt saw and ground to a thickness of 100 μm using the Exakt micro-grinding system and polished on the Monopoly 2000 (Bueler, Coventry, UK). Prior to histomorphometric analysis of the sections they were stained with Toludine Blue for 10 minutes, which stains fibrous tissue blue.

2.2.5.5 SEM analysis

All DMEM was removed from the samples by washing three times with PBS. The samples were fixed with 2.5% glutaraldehyde (Agar Scientific R1011) in 0.1M sodium cacodylate (BDH Chemicals 30118) buffer for 2 hours. The samples were rinsed twice for 5 minutes in 0.1M sodium cacodylate buffer in distilled water to remove any gluteraldehyde. Dehydration of the samples involved a number of processing steps. Samples were covered with 1% osmium tetroxide (Agar Scientific R1022) in 0.1M sodium cacodylate buffer for 60 minutes. The Osmium was then discarded. The samples were rinsed three times for 5 minutes in 0.1M sodium cacodylate. This was followed with incubation of the samples in 1% tannic acid (Sigma T0125) in 0.05% sodium cacodylate buffer for one hour. The tannic acid was discarded and samples were rinsed for 10 minutes, four times in

0.1M sodium cacodylate. Samples were further dehydrated in increasing concentrations of ethyl alcohol for 5 minutes. Each step was repeated once with concentrations at: 20%, 30%, 40%, 50%, 60% and 70%. Absolute alcohol was added to the samples twice for ten minutes at each of the following concentrations 90%, 96% and 100%. Finally samples were placed into a consecutive series of fresh vials containing hexamethyldisiazane (HDMS) (Agar Scientific) for 2 minutes at a time. Samples were air dried and mounted on aluminium stubs. All samples were fixed and gold sputter coated using an EMITECH K550 machine. Pictures of these samples were taken using a JEOL JSM-5500LV.

2.2.6 Statistical analysis

Cell metabolism measured by Alamar Blue^{TM} values for the force groups (0kN, 3kN, 6kN and 9kN) at day 5 (the 1st day after impaction) and day 10 (the 6th day after impaction) were analysed by use of univariate analysis of variance (ANOVA). The statistical software SPSS (SPSS v10.1, SPSS Inc, Chicago, USA) was used for the analysis. The absorbance values were used as the "dependent variable" and the impaction forces were used as "group" variables. If the global analysis of variance was significant at the 5% level, a post hoc pairwise comparison Dunnett's Test was carried out to examine any differences among the impaction forces.

2.3 RESULTS

2.3.1 Harvesting MSCs

2.3.1.1 Observations of MSCs in vitro

After isolation of the mononuclear fraction of cells from the Ficoll® buffy layer, primary cell populations appeared to be densely populated with cells of different sizes that remained rounded in suspension. Adherent cells could be observed after one day or two days. Initially isolated and adherent cells displayed a fibroblastic morphology of one nuclei and elongated spindle-like processes (figure 2.3a). More cells became adherent at different timepoints over a period of four days. Within four days, most of the cells in suspension were cleared from the flasks during medium changes (figure 2.3b). During this time, and usually after 7 days, it was observed that the cells had started to form colonies of varying cell numbers (figure 2.3c). The numbers of cells in each colony continued to grow until subconfluence at days 10-14 (figure 2.3d). At this point the cells were passaged to prevent overgrowth of the cells. The morphology of the cells remained consistent up to at least 12 passages.

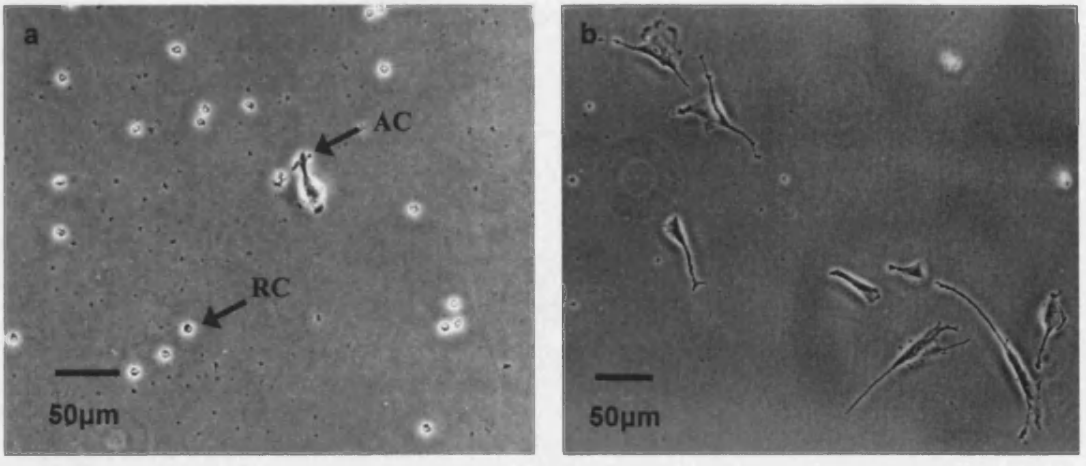


Figure 2.3 Cells in culture at passage P0. **a** Image of Ficoll® isolated cells after 2 days in culture. The label AC indicates an adherent cell with spindle-like processes, RC (rounded cell) indicates a cell in suspension; **b** Day 4. There are no cells in suspension, all cells in the field are adherent to tissue culture plastic.

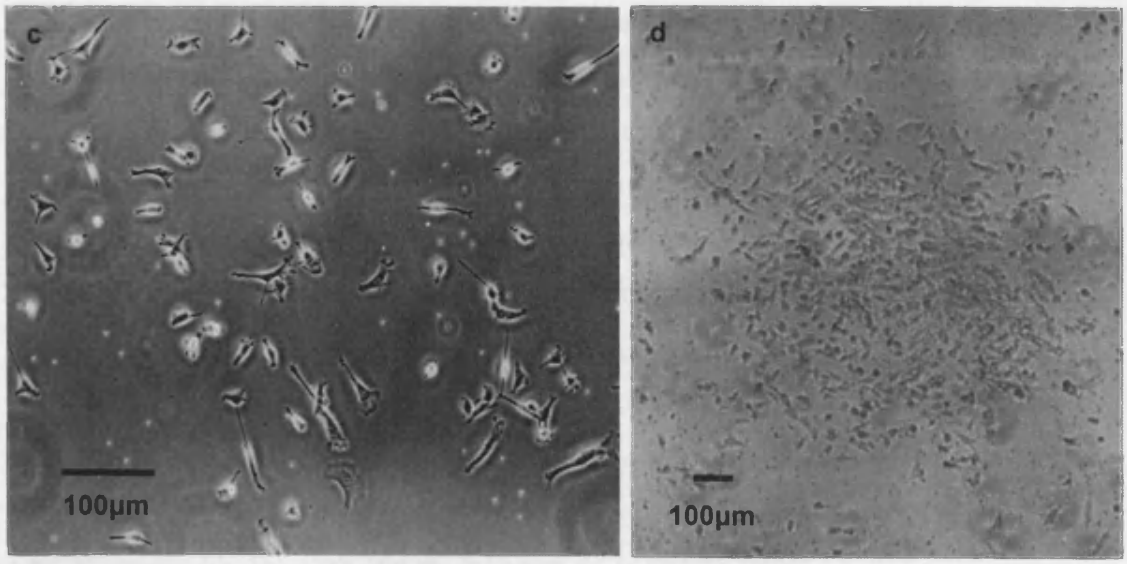


Figure 2.3c Day 10. There was an increase in the number of adherent cells. **D** After 14 days at P0 cells were clustered in round colonies.

2.3.1.2 Growth curve for cells in monolayer

Figure 2.4 shows the growth rate of the MSCs. A peak in the growth rate was observed on day 4 after seeding. Consequently impaction was carried out on day 4 because the cells were still in the growth phase.

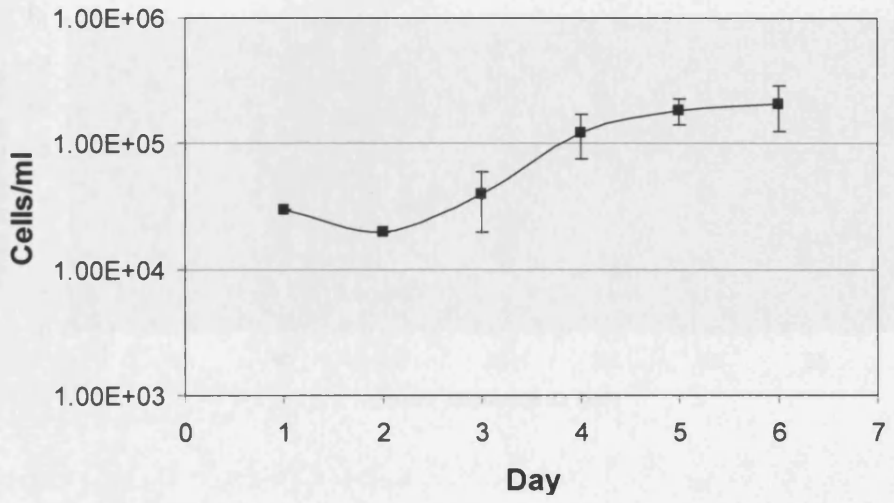


Figure 2.4 Graph to show the number of live cells in monolayer at daily intervals

2.3.2 Determining impaction forces

2.3.2.1 Normal forces used in surgery

Figure 2.5 shows distal impaction forces measured during a single surgery from the load washer mounted onto the Exeter slap hammer. In this graph each bar represents a single impaction and shows the force detected by the load washer positioned within the hammer. Averaged measurements of intra-operative forces used by 9 surgeons during impaction occurred are in the range of 1.8 to 8.6kN after calibration is taken into consideration. These forces were calibrated to the forces impacted onto the allograft. The average forces that allograft received during revision THR were in a range between 3kN and 6kN with the highest forces reaching 9kN. Relating the hammer forces measured during surgery to the forces on the graft showed that one third of the impaction force detected at the hammer was transmitted to the graft (figure 2.1b and 2.5) (Phipps 2004).

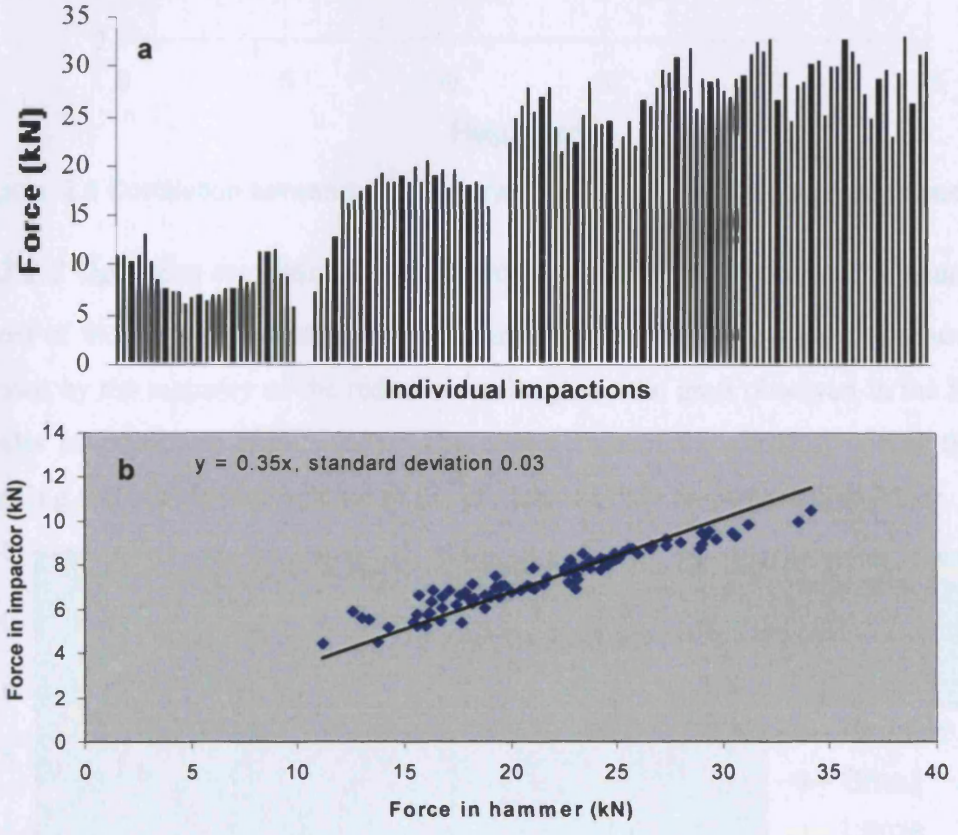


Figure 2.5a Impaction forces measured in the hammer during a revision total hip replacement are three times greater than those received by the graft **b** Relationship between force in hammer and force in impactor, measured in an experimental set-up with two load washers (Phipps 2004).

2.3.2.2 *Impactor drop heights to replicate forces used in a normal surgery*

Figure 2.6 shows the correlation between a range of impaction forces and drop heights of the impactor. These are: 2.5cm for a force of 3kN, 5.2cm for a force of 6kN and 10cm for a force of 9kN.

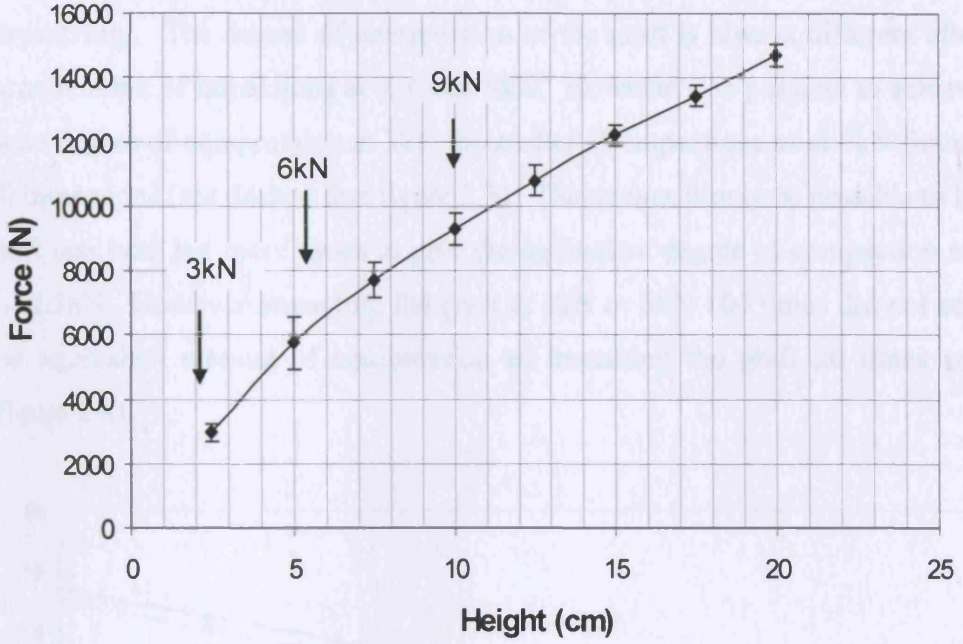


Figure 2.6 Correlation between range of graft forces and drop height of the impactor

2.3.2.3 *Optimum number of impact cycles to achieve maximum compaction*

Most of the compaction of allograft occurs with the first 20 cycles of impaction as shown by the majority of the reduction in height of the graft observed in the first 20 cycles of impaction (figure 2.7). The compaction of the allograft occurs through packing and plastic deformation of the particles as they are squeezed together.

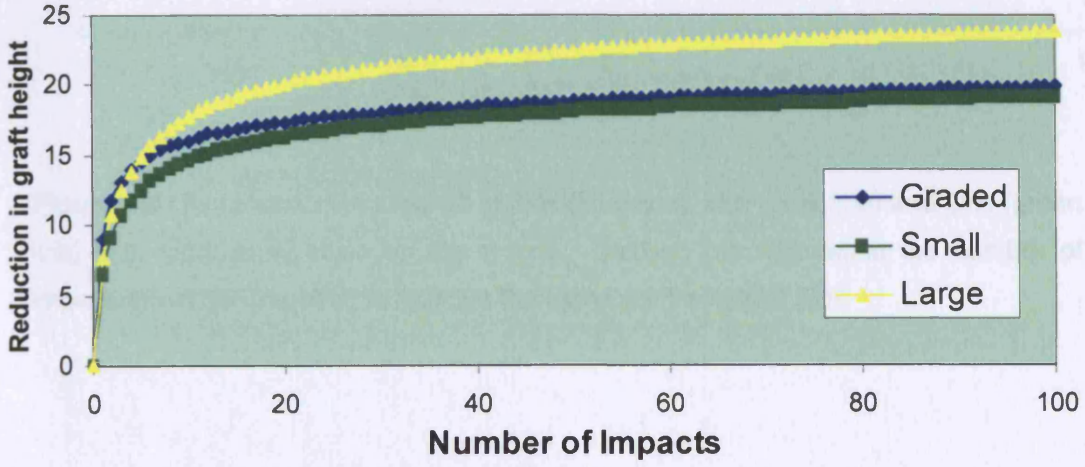


Figure 2.7 Average reduction in height of morsellised allograft in Delrin cylinders during 100 impactions at 3kN (Phipps 2004)

2.3.2.4 Results showing the collapse of graft under different loads

Impaction of the graft results in a more compact graft as the force increases. After 20 impactions the graft is 11.6mm, 11.2mm and 8.7mm in height at 3, 6 and 9kN respectively. The degree of compression of the graft is always different after the same number of impactions at 3, 6 and 9kN. However it is possible to achieve the same degree of compression at 3kN force after 30 impactions as at 6kN force after 20 impactions (see dashed line figure 2.8). This means it may be possible to hit the graft less hard but more times to give the equivalent degree of compaction at 6kN as at 3kN. However impacting the graft at 3kN or 6kN 100 times did not achieve the equivalent amount of compression as impacting the graft 20 times at 9kN (figure 2.8).

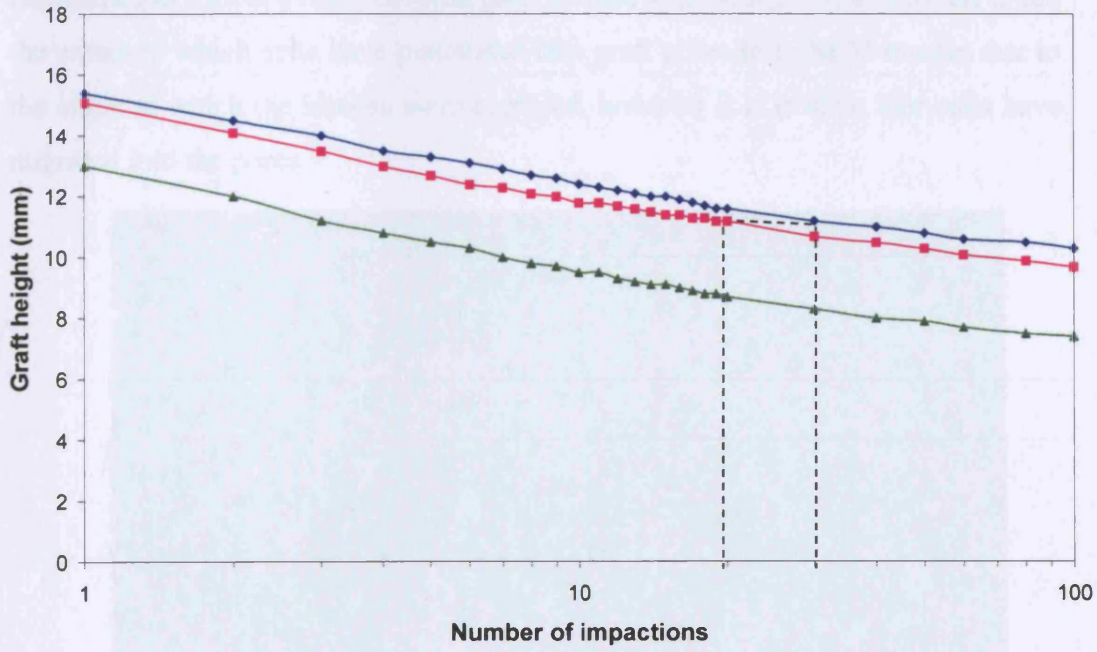


Figure 2.8 Compression of allograft at 3kN (blue line), 6kN (pink line) and 9kN (green line) with logarithmic scale on the x axis. Dashed line represents the number of impactions at 3kN required to achieve the same graft height at 6kN.

2.3.3 Determination of seeding technique

2.3.3.1 Cell seeding results: SEM and resin images

Figure 2.9a demonstrates a granule of graft in a control where no cells were seeded. Figure 2.9b shows density and suspension seeded cells on graft. Density seeding resulted in large clumps of cells overlapping with one another on the graft (figure 2.9c). There were also areas of density seeded graft that were entirely devoid of cells (figure 2.9d). On suspension seeded graft areas of un-colonised bone could be identified, adherent cells were sub-confluent, in close contact with the surface of the allograft and more evenly distributed (figure 2.9d). Although these results are qualitative they do demonstrate that suspension seeding culminates in a more evenly covered graft surface with MSCs. It is difficult to tell the extent to which cells have penetrated into graft pores from SEM images due to the angle at which the images were captured, however it is evident that cells have migrated into the pores.

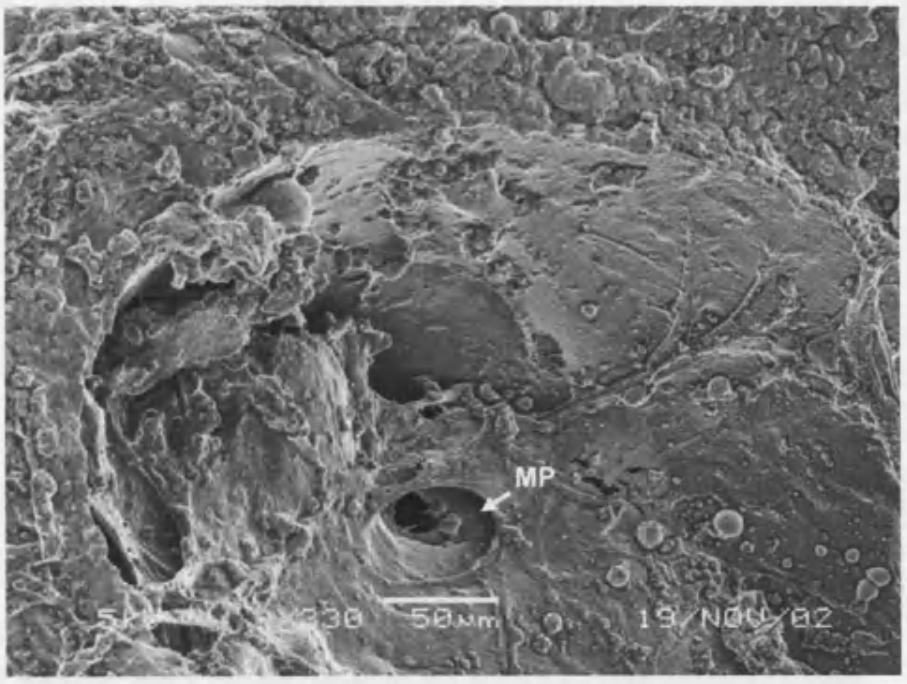


Figure 2.9a Control sample of an allograft granule showing a micropore and a surface of varying roughness.

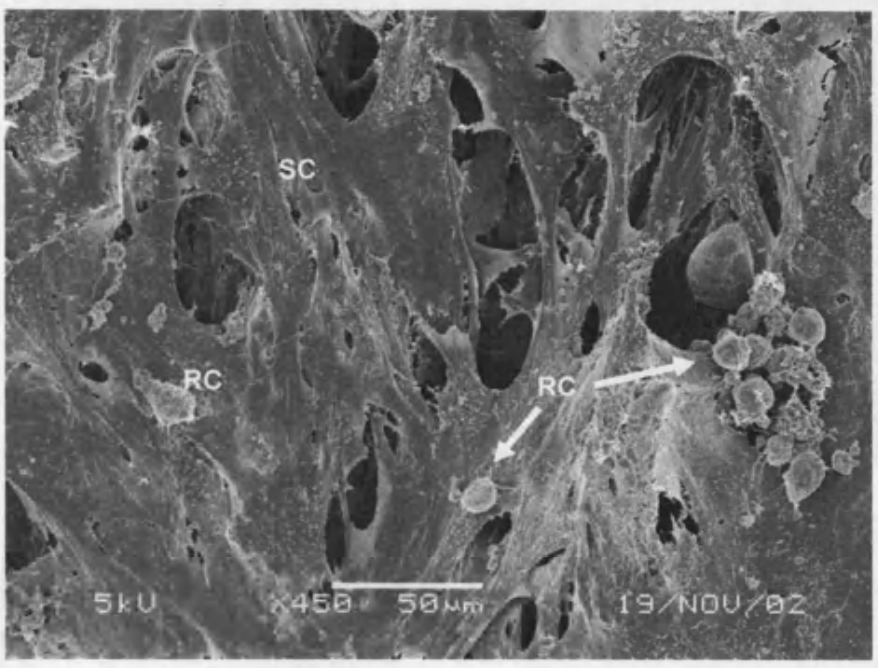


Figure 2.9b Density seeded samples. Spread cells (SC) have adhered to one another in layers over the surface of the allograft granule. RC are rounded cells that were beginning to adhere to the surface of spread cells just prior to fixation.

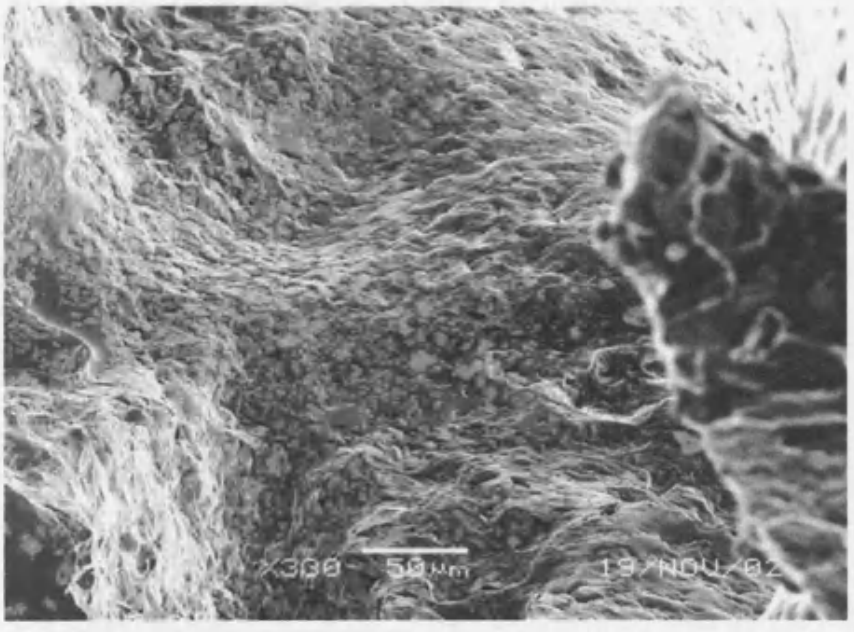


Figure 2.9c Density seeded sample. No cells were observed in this area of allograft granule.

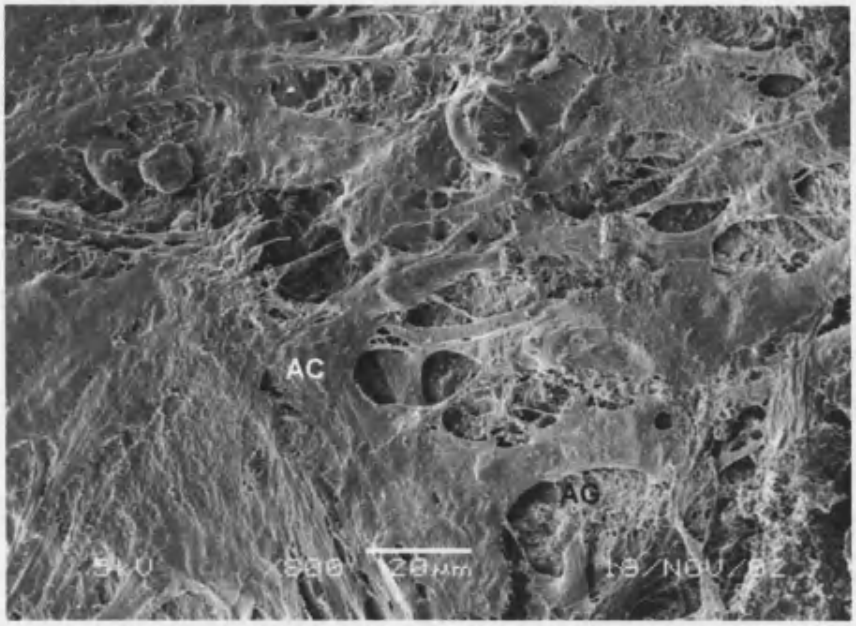


Figure 2.9d Granule of allograft from suspension seeded samples. Adherent cells (AC) are subconfluent and in close contact on the surface of the allograft. There is some allograft (AG) that is not covered by cells.

2.3.4 The effect of different impaction forces on MSCs in allograft conduits

2.3.4.1 Alamar Blue™ results

Figure 2.10 shows that the MSC growth curve for 0kN has a lag period and growth phase. Compared with the control (0kN) at day 2 (the 1st day after impaction), the impaction at 3kN force showed no significant difference ($p=0.06$), while both 6kN and 9kN showed a significant difference respectively ($p<0.001$). However, the MSCs recovered to different levels of metabolism after impaction. At day 7 (the 6th day after impaction), both 3kN and 6kN showed no significant differences compared with 0kN ($p=0.904$ for 3kN and $p=0.095$ for 6kN), but for the 9kN group, the difference was still highly significant ($p<0.001$). The images in figures 2.11 and 2.12 show samples that are representative of cells attached to graft after impaction at different impaction forces.

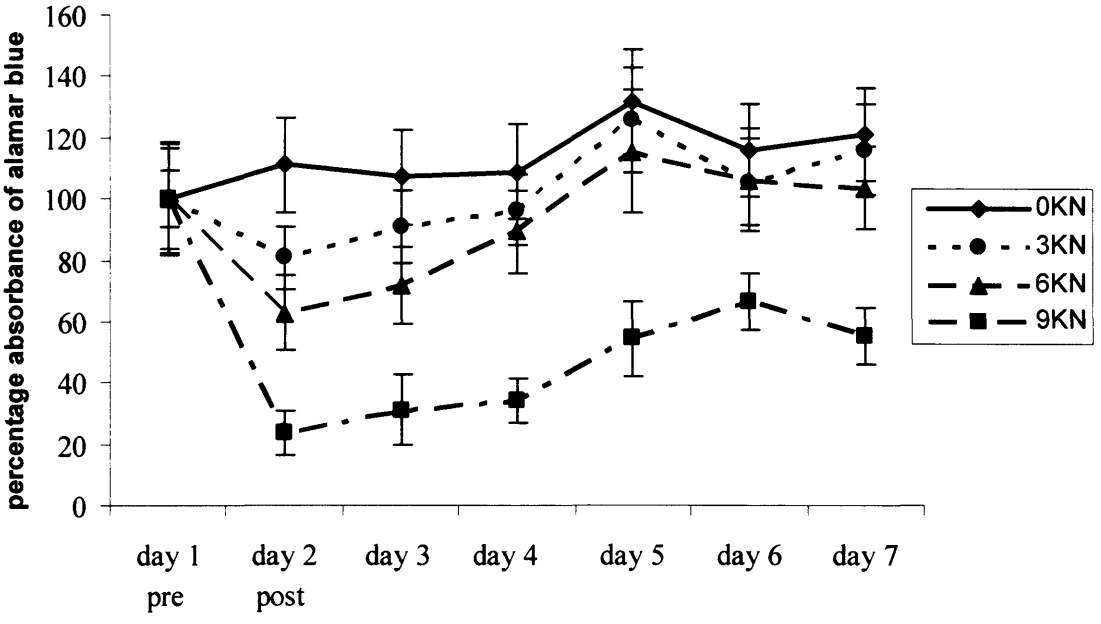


Figure 2.10 Percentage absorbance of Alamar Blue™ on cells in impacted and non impacted graft.

2.3.4.2 SEM results to show effect of impaction on suspension seeded MSCs

Figures 2.10a-d show MSCs suspension seeded on allograft at 2.5 and 72 hours before impaction and demonstrate spreading of the MSCs on the allograft surface over time. Figure 2.11a and 2.11b show that even after 2.5 hours cells have already spread out on the allograft surface. Rounded cells can also be seen (figure 2.11a). After 72 hours MSCs are also spread on the allograft surface (figure 2.11c), however, there are still rounded cells present (figure 2.11d). Rounded cells at either time-point may suggest cells have already begun dividing or that the cells are adhering from the original suspension. Figures 2.11e-g show MSCs suspension seeded onto allograft on day 6 in the impacted and unimpacted control groups. In the 0kN control (figure 2.11e) cells have continued dividing after 6 days as evidenced by numerous rounded cells on the surface of the graft. At 3kN force cells are spread on the surface of the graft after 6 days (figure 2.11f); however some of the cells at the surface are fractured across the body of the cell or at focal points. This may be as a result of impaction or abrasion of the granules against each other during tissue culture processing. At 6kN cells appear stretched between depressions in the graft (figure 2.11g) and at 9kN only cell processes can be observed suggesting cell damage. These observations are qualitative but indicate an increasing level of detriment to the cells with force of impaction, which concurs with the Alamar Blue™ assay for viability (figure 2.10).

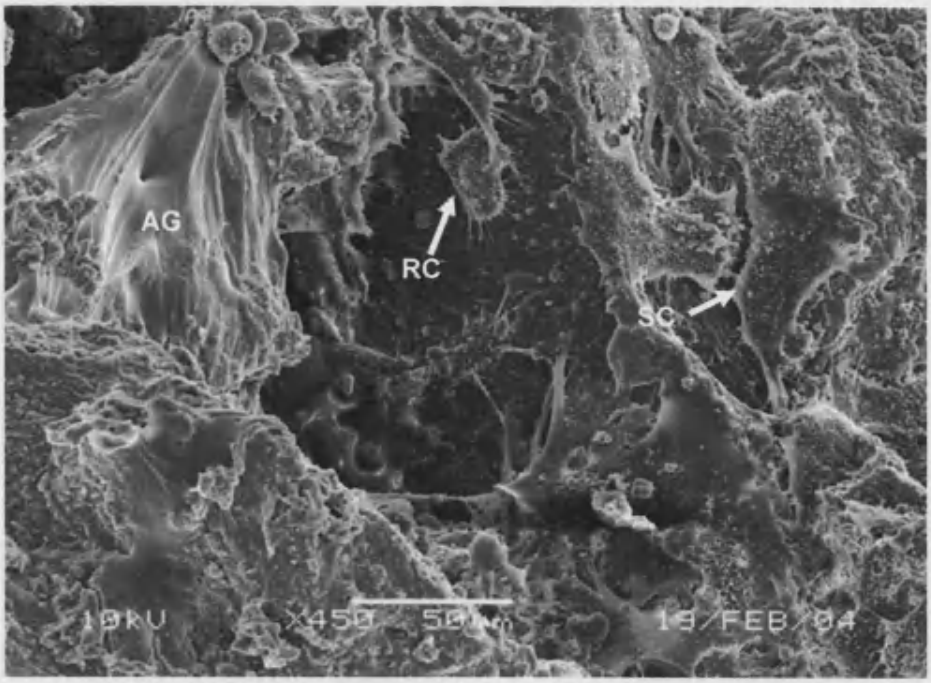


Figure 2.11a Cells on allograft after 2.5 hours. There are some rounded cells (RC) that appear to be the latest cells to adhere to the surface of the allograft (AG), and spread cells (SC). These could also be cells dividing from nearby cells or those underneath.

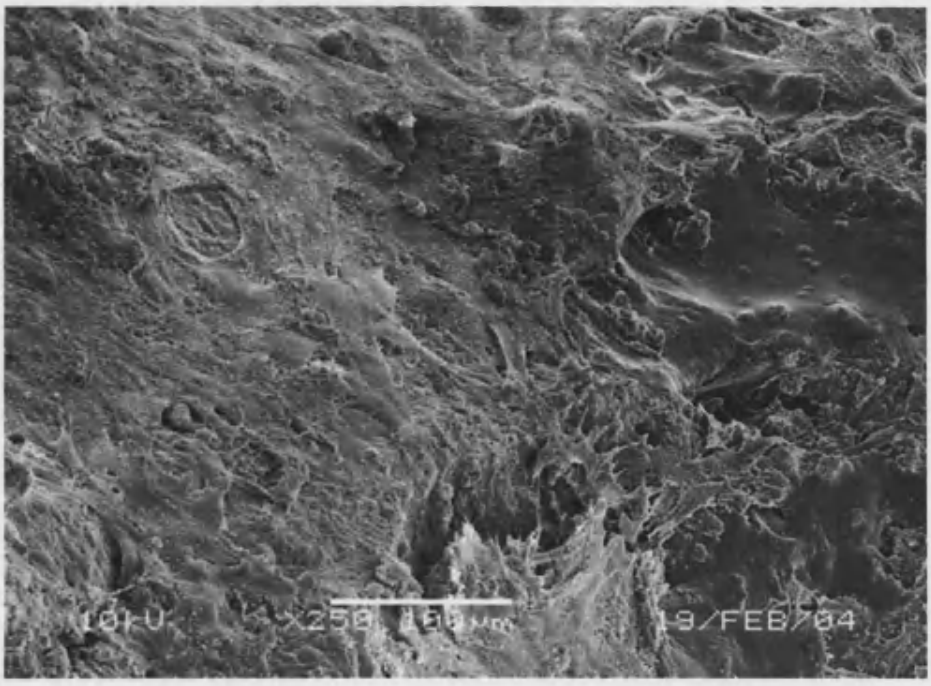


Figure 2.11b Cells on allograft after 2.5 hours. Most of the allograft surface is covered with spread cells.

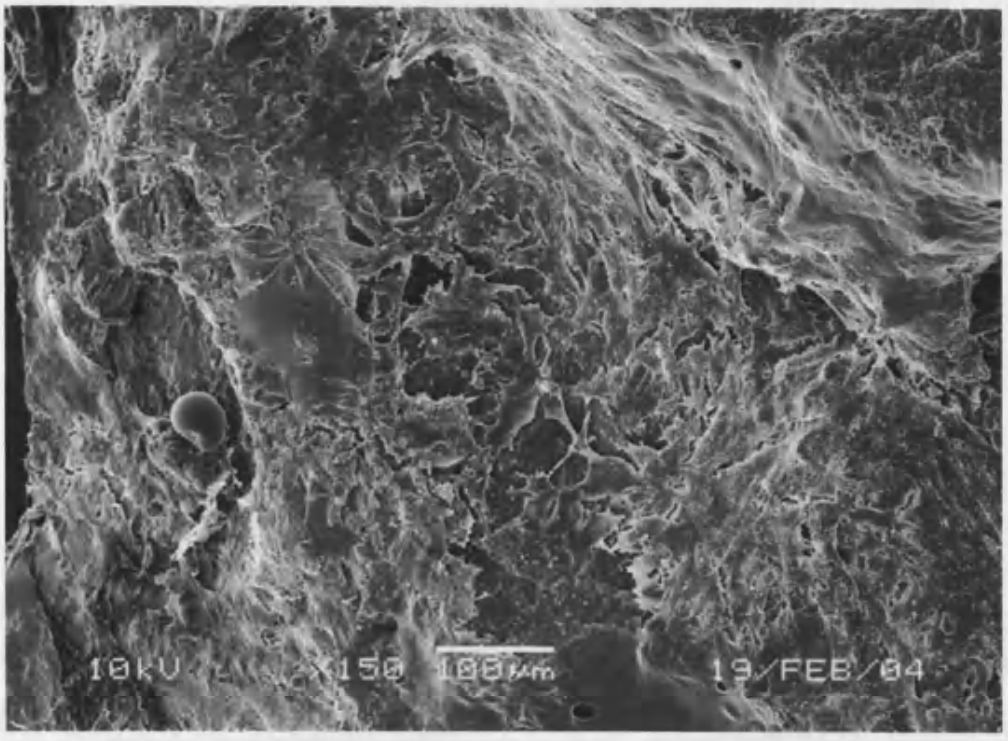


Figure 2.11c Cells on allograft after 72 hours. Cells cover the surface of the graft and are spread cells.

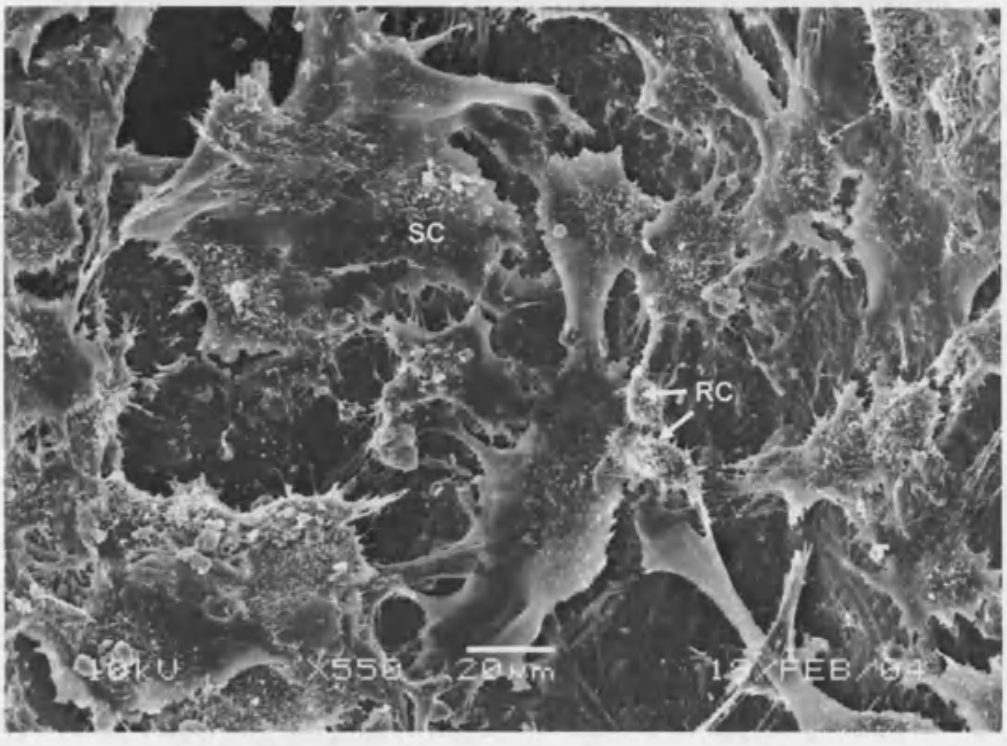


Figure 2.11d Cells on allograft after 72 hours. Cells cover the surface of the graft. There are spread cells (SC) and rounded cells (RC).

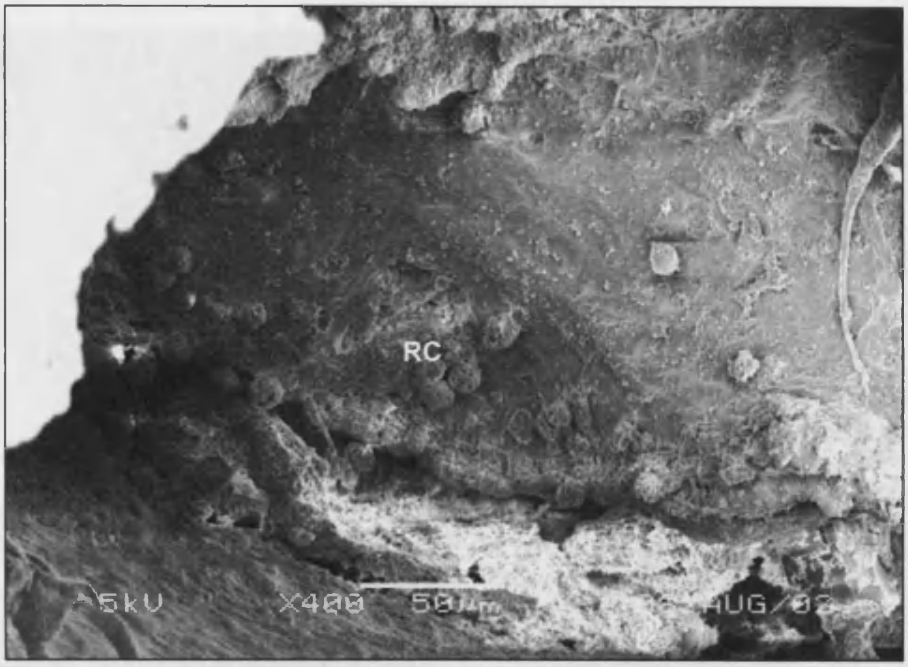


Figure 2.11e Cells at 0kN impaction after 6 days covering a large proportion of the allograft granule surface.

Figure 2.11f Cells at 3kN after 6 days. Cells appear light grey, covering the entire surface of the graft within view. Cells that have adhered on top of these lighter cells appear dark grey. There are also some rounded cells (RC) which are either arrested in growth or more likely to be in the process of dividing and growing on the surface of other cells. One of the processes extending from the cell appears to have broken away from the main body of the cell (BC).

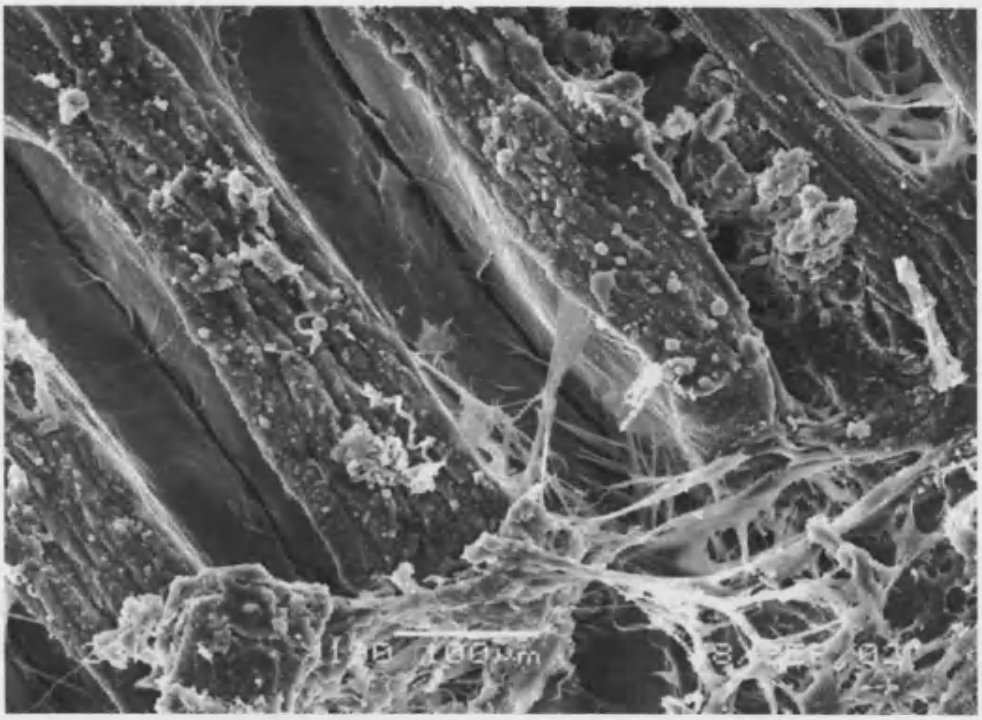


Figure 2.11g Shows a 6kN sample after 6 days. Cells are present on the surface of the graft and are stretched between depressions in the graft surface.

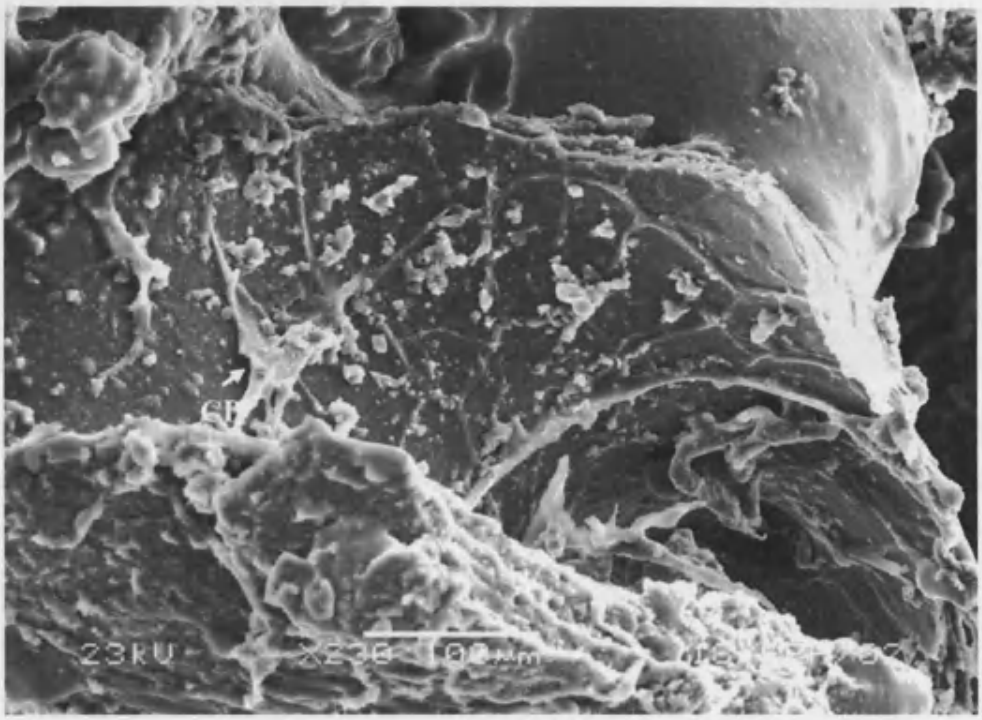


Figure 2.11h Sample from 9kN group after 6 days. Cell processes can be observed. There are no cells flattened against the surface of the graft.

2.3.4.3 Histology to show effect of impaction on cells.

Figure 2.12a-d shows MSCs adherent to the surface of the allograft on day 6 in the impacted and unimpacted control groups.

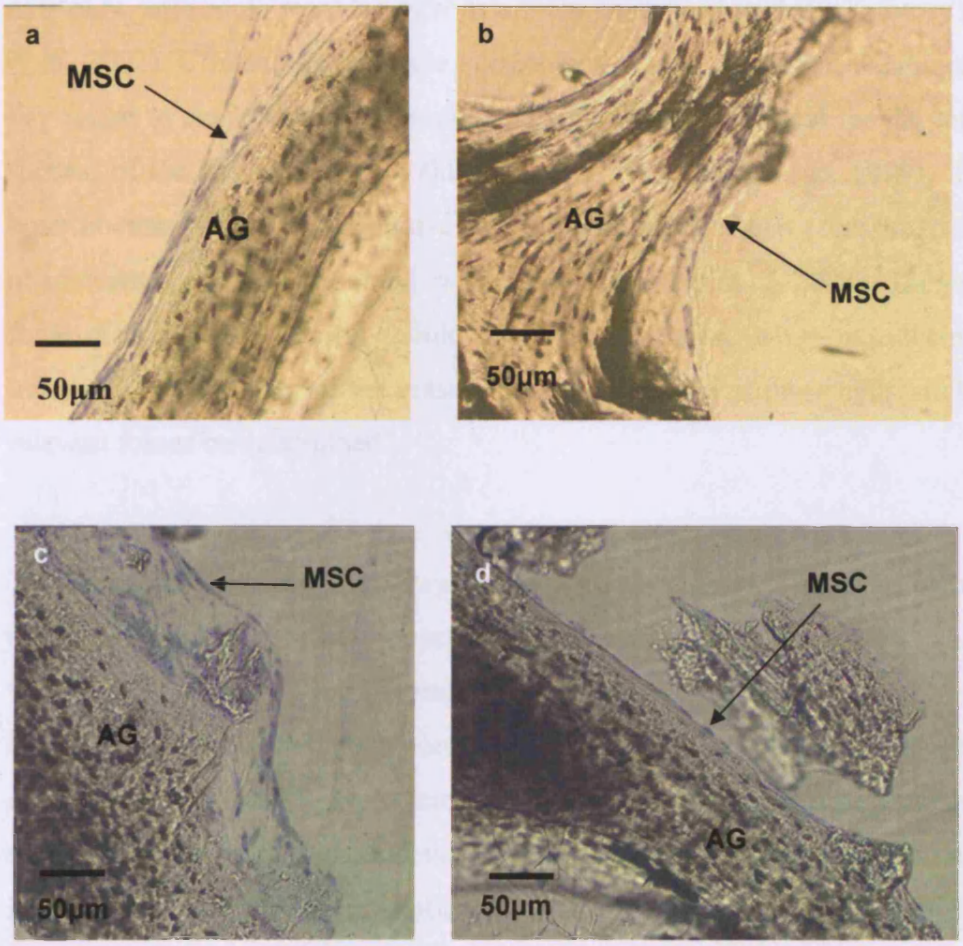


Figure 2.12a-d Histology images of allograft groups stained with toluidine blue. **A** 0kN impacted group shows layers of cells on the surface of the graft indicated by darkly stained nuclei and fibrous tissue stained lighter blue, **b** 3kN, **c** 6kN, **d** 9kN

2.4 DISCUSSION

In revision total hip replacements, impaction occurred has been widely used to fill bone defects and to provide sufficient bone stock for implant fixation. Impaction forces are variable between surgeons, and cases, and have been recorded in a range of 3-6kN, with some reaching 9kN in a study performed in this laboratory (Phipps *et al.* 2002). Clinical studies have shown that a good impaction technique is the key factor to achieving initial implant stability which is critical for the long-term success of the implant (Tägil 2000; Gie *et al.* 1992; Wang *et al.* 1999). Femoral bone fractures as well as excessive stem migration have been noted in some cases of impaction occurred (Leopold *et al.* 1999; Cabanela *et al.* 2003). Therefore the force of impaction allograft should not be compromised while introducing MSC treatment. This is one of the reasons that the viability of these cells at clinically relevant forces be determined.

The adapted slap hammer is easy to use and sterile, and is capable of instantly measuring and recording impaction force during revision THR. The huge variations of impaction forces measured from different surgeons suggested that the impaction forces should be monitored and regulated. Allograft can then be consistently impacted to avoid unnecessary bone fracture and excess migration of the stems. Griffon D.J. *et al.* developed an ovine model to evaluate the effect of impacted biomaterials in unicortical defects (Griffon *et al.* 2001). Differences were found between sites of implantation using a subjective grading system, with greater bone healing in distal femoral sites compared to proximal tibial sites. Proximal femoral defects extended to the far cortex, which may have affected the degree of compaction. Therefore the distal femoral site was confirmed as a better model. However, the impactor used to impart the forces to the graft was based on a design by Brewster *et al.* (Brewster *et al.* 1999). The energy of impaction was determined by using a slap hammer to impact graft into a plastic femur on a force plate. A plastic femur is not representative of the force distribution in a human femur during surgery. The direction of the hits are different, with forces directed downwards onto the force plate and sideways in surgery, due to the shape of the impactor, which will affect the force going through the graft. The measurements

made by Kirsty Phipps used a load cell within the hammer and in the proximal impactor embedded in the graft (Phipps 2004), which is more accurate than forces measured directly from the impactor on a force plate. The forces used in this Chapter are also more accurate than Griffon *et al.* because they were replicated at a range of forces from several surgeons and Griffon *et al.* uses only one plastic femur. A greater amount of impaction will decrease the surface area of graft as graft pores fracture and collapse, allowing for greater osteoconduction because particles are touching each other. However if the graft is too compressed as a result of impaction, this may also affect the vascularisation of the graft which is essential for bone formation and remodelling, as a more compressed graft may resist penetration of blood vessels. A study by Tägil showed the latter. Graft was compressed to different volumes in a bone conduction chamber and implanted into the tibial metaphyses of 17 rats. Unimpacted allograft was implanted as a control on the contralateral side of each rat. Impaction led to a decreased ingrowth of bone (Tägil and Aspenberg 1998). This may have been because the compression of the graft was not related to clinical impaction forces applied under surgical conditions. The stability of an implant may also be compromised depending on the level of impaction of the graft. Initial stability of the implant can be attained by impacting graft around the implant to the extent that it can withstand the load on the femur. If impaction is inadequate it can result in lack of initial stability, resulting in migration of the prosthesis and subsidence (Tägil and Aspenberg 1998; Gie *et al.* 1992).

Many studies showed that MSCs can be differentiated into several cell lineages, which include osteoblasts (Jessop *et al.* 1994; Muraglia *et al.* 2000). Several animal studies demonstrated that MSCs seeded onto adequate scaffold materials can repair segmental defects and regenerate new bone in a fracture model (Blokhuis *et al.* 1999; Arinzeh *et al.* 2003; Kadiyala and Jaiswal 1997; Bruder *et al.* 1998b; Chistolini *et al.* 1999). However, most of the scaffolds used in these studies were solid blocks with a porous structure (Ripamonti 1991; van Eeden and Ripamonti 1993; Harris and Cooper 2004). In a study by Mushipe *et al.* the suvivourship of MG63 cells on allograft after impaction was tested *in vitro* using a replica of an acetabular cup. DNA content of cells removed from the graft was measured after each impact from the modified impactor. After 5 impacts 21% of

the cells were viable relative to the control. This study shows that some cells survived impaction (Mushipe *et al.* 2006). However the cell line was a transformed osteoblastic cell line, which could not be translated to a clinical situation, and may behave differently to MSCs. The impaction forces were determined using a wooden acetabulum cup model and were tested using only one surgeon. My study established that the force of impaction varies widely between surgeons and I used a range of forces to test MSC viability.

This study is the first *in vitro* attempt to examine the effect of impaction forces on MSC viability. Proliferation was measured using the Alamar Blue^{TM} assay. This assay incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell proliferation. This assay is a reliable method to assess cell viability and proliferation, and has a very high agreement with flow cytometry in indicating the number of living and dead cells in cultures (Reis *et al.* 2004). The results from the study showed that the viability of the MSCs is associated with the amount of impaction force used during surgery. A minimal effect on MSC viability was observed up to 6kN impaction forces, but when using 9kN, the viability of the cells was significantly reduced. The study suggests that it is feasible to use MSCs addition to the allograft under normal impaction forces during revision THR and excessively high impaction forces such as 9kN should be avoided. It is possible to achieve the same degree of compression at 3kN force after 30 impactions as at 6kN force after 20 impactions. The viability of the cells may possibly be maintained if they are hit less hard but a greater number of times. Increasing the number of hits at a lower force is not applicable in this instance because the cells recover at the 6kN impaction. However, the same degree of compression of the graft could not be achieved in the 3kN group or the 6kN group as in the 9kN group, even after 100 impactions. So it appears that survival of cells after impaction is a consequence of the forces used. It is important to remember that the force used must achieve stability of the graft. However it appears that different surgeons use variable amounts of force. It may be that by using a larger number of impactions at a lower load, the graft is as stable as that achieved using fewer impactions with greater force. If MSCs are used for impaction grafting surgeons should be encouraged to use a higher number of impactions with reduced force.

2.5 CONCLUSION

The results demonstrated that MSCs survived normal impaction forces, but will be affected if the force on the graft is above 6kN. The hypothesis that MSCs seeded onto allograft scaffolds will survive normal impaction forces that was presented in the introduction of this chapter has been proved. In conclusion addition of MSCs to the allograft scaffold followed by impaction grafting will not destroy the MSCs and therefore this technique has potential in regenerating new bone to repair bone defects in rTHRs.

CHAPTER 3: *In vitro* Impaction of MSC derived Osteoblasts on an Allograft Scaffold

3.1 INTRODUCTION

The *in vitro* study in Chapter 2 showed that MSCs could survive normal impaction forces used in revision total hip replacements ranging from 3kN to 6kN (Korda *et al.* 2006; Phipps *et al.* 2002). MSC metabolism decreased with increased force one day immediately after impaction at 3, 6 and 9kN. By day 7 MSCs impacted at 3 and 6kN had recovered to levels of metabolic activity not significantly different from the 0kN control, although the 6kN group was less metabolically active than the 3kN group. However the 9kN group remained significantly less metabolically active after day 7 compared to the 0kN group.

The general introduction to this thesis discussed the possibility of using osteoblasts or osteoblasts derived from MSCs in combination with impaction occurred as an alternative to MSCs. Osteoblasts may be an improvement on using MSCs as they are a more mature phenotype and therefore committed to the osteogenic lineage, potentially reducing the time between lineage progression and therefore bone formation *in vivo* (Bruder *et al.* 1994; Kadiyala and Jaiswal 1997; Bruder *et al.* 1998a). The source of more rapid bone formation from these cells could come from a combination of effects. Growth factors such as OSF-1 (osteoblast-stimulating factor) are produced by osteoblasts, suggesting that it influences the behaviour of the same osteoblasts that produce it locally. OSF-1 may also influence osteoblastic activity by recruiting osteoprogenitor cells or MSCs from the indigenous population to induce osteoinduction. Evidence to support these contentions comes from a study by Yang, X *et al.* where human cells combined with OSF-1 on a PLGA scaffold implanted subcutaneously in diffusion chambers in athymic mice demonstrated new bone formation, whereas control chambers without OSF-1 did not. OSF-1 was also chemotactic and stimulated differentiation and mineralisation of human MSCs on PLGA scaffolds *in vitro* (Yang *et al.* 2003). Bruder *et al.* states that chondrocytes are not required for osteogenesis, which suggests that cells are recruited from the native population by osteoinductive factors (Bruder and Caplan 1990a). TGF-β is produced predominantly by osteoblasts which have the largest number of TGF-β receptors. TGF-β stimulates proliferation of MSCs in the periosteum during fracture healing. Other factors

such as BMPs, which have been discussed in the general introduction of this thesis, are also produced by osteoblasts as well as other cell types (Solheim 1998).

In vivo evidence to support the use of osteoblasts in accelerating and increasing osteogenesis compared to MSCs was found by Yoshikawa *et al* (Yoshikawa *et al.* 1996). Syngenic rat MSCs seeded onto HA blocks and subcultured for 14 days either in osteogenically supplemented or standard control media were implanted subcutaneously. After 8 weeks the osteogenically treated samples produced bone and control samples with undifferentiated MSCs did not (Yoshikawa *et al.* 1996). Breibart *et al.* derived osteoblasts by differentiating periosteal cells and seeded them onto polyglycolic acid scaffolds (Breibart *et al.* 1998). Bone formation in this group was compared to that in polyglycolic acid scaffolds without osteoblasts implanted in rabbit calvarial defects. New bone was found in osteoblast loaded sites whereas only fibrous tissue was found in the group with MSCs (Breibart *et al.* 1998). Additional examples of the relative benefit of using osteoblasts compared to other cell sources include studies comparing osteoblasts with fresh bone marrow (Kadiyala and Jaiswal 1997; Bruder *et al.* 1998a; Bruder and Fox 1999; Kruyt *et al.* 2003). These studies suggest that osteoblasts may expedite the process of bone healing relative to other cell sources.

Mature osteoblasts would be inefficient in bone tissue engineering as they are more difficult to obtain and undergo limited expansion *in vitro* (Bruder and Fox 1999). This also applies to osteoblastic cells derived from the marrow element without expansion. Although the experiments cited above suggest the feasability of using fresh bone marrow, the presence of osteogenic precursors will vary widely between patients and may be depleted in the elderly as a result of aging (Bruder and Fox 1999; Caplan 1994). Introducing committed osteoblasts may directly induce bone, but these osteoblasts would only be able to recruit a limited number of osteoprogenitors in a patient whose host tissue bed has been compromised due to smoking, diabetes, irradiation, aging or osteoporosis (Bruder and Fox 1999).

MSC derived osteoblasts allow a degree of control whereby the desired quantity of MSCs can be multiplied prior to differentiation into osteoblasts. In addition, the stage of differentiation can be controlled. Monoclonal antibodies have been used

to target markers for osteoprogenitor cells at discrete developmental stages of the osteogenic lineage in embryonic chick periosteum (Bruder and Caplan 1990a) and tibia (Bruder and Caplan 1989). Osteoprogenitors proceeded through a series of lineage steps marked by different antibodies until they stop dividing and begin to deposit osteoid, at which point they are termed **'secretory osteoblasts'**. The subsequent, more mature cell types in the osteogenic lineage do not secrete osteoid matrix (Bruder and Caplan 1990a; Bruder and Caplan 1989; Bruder and Caplan 1990b). **'Secretory osteoblasts'** can be selected for their osteoid producing properties during differentiation *in vitro* as they stain positive for ALP (Bruder *et al.* 1998a; Bruder and Caplan 1990a; Bruder and Caplan 1989; Bruder and Caplan 1990b). The osteoblasts in this chapter were selected for impaction at the peak of ALP expression to ensure they had not surpassed the secretory osteoblastic stage of differentiation. All cells differentiated from MSCs are termed **'osteoblasts'** throughout the rest of this thesis.

Although osteoblasts can shorten the time between cell implantation and bone formation, their response to different impaction forces was unknown. This chapter will investigate whether MSC derived osteoblasts can survive normal, clinical impaction forces. Comparisons will be made between the viability of osteoblasts and the effect of the same impaction forces on MSCs investigated in the previous chapter.

Aim

To test the viability *in vitro* of osteoblasts derived from MSCs which were seeded onto the allograft and impacted at a range of forces obtained during rTHRs.

Hypothesis

Osteoblasts derived from MSCs seeded onto allograft scaffolds will survive normal impaction forces used during rTHRs.

3.2 MATERIALS AND METHODS

3.2.1 Differentiation of MSCs into osteoblasts

3.2.1.1 MSC culture

MSCs were isolated from bone marrow aspirates of 6 sheep designated for the study. The cells used in the study were spindle shaped in appearance once adherent to tissue culture plastic and displayed this morphology throughout culture. The MSCs were expanded in culture until they were at passage 2 to achieve adequate numbers for cell seeding onto allograft. After trypsinisation $1x10^6$ cells/ml were resuspended in 1ml of 10% Dimethylsulfoxide (DMSO, D5879, Sigma-Aldrich Ltd, Fancy Road, Poole, Dorset, UK) in FCS (First Link, West Midlands, UK). The cell suspensions were then transferred to cryovials in a 'Mr Frosty' 5100 Cryo 1°C freezing container (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, UK) containing isopropan-2-ol which was placed in a -70°C freezer for 24 hours. The vials were then transferred to liquid nitrogen until resuscitation.

3.2.1.2 Resuscitation of cryopreserved cells

Cryovials of MSCs and primary sheep dermal fibroblasts (SDFs), made from skin explants, were incubated in a waterbath at 37°C until the ice had thawed. Thawed cells were resuspended in 1ml of growth media which was warmed to 37°C in the waterbath before being added. The cells were allowed to come to room temperature for 5 minutes before addition of more growth media. The cells were then added to a universal tube and doubling volumes of warmed medium were added to the cells until 16ml total volume was reached with 5 minute equilibration periods between each addition. The cell suspensions were centrifuged at 2000rpm for 5 minutes. The cell pellets were resuspended in 2ml of growth media, transferred to culture flasks, designated passage 3 and cultured in accordance with the protocols described in chapter 2 after resuscitation of the cells from frozen.

3.2.1.3 Osteogenic differentiation of MSCs

Cells were trypsinised from flasks and resuspended in flasks at $7x10^4$ cells/ml into T225 flasks. After 7 days MSCs and SDFs had reached optimal cell density for

osteogenic differentiation at 90% confluence. At this point the growth media was supplemented with osteogenic supplements to make osteogenic media (OM). The OM was made up of DMEM (DMEM, Sigma D-6429), with 10% FCS (First Link, West Midlands, UK), penicillin / streptomycin (1000iu/ml; 100μg/ml) (GIBCO Paisley, UK) supplemented with 10mM β-glycerophosphate (Sigma D1756), 50μg/ml Ascorbic Acid (Sigma A4544), and 1 x 10^{-8} M Dexamethasone (Sigma, D-2915), (Goldstein 2001). All media was changed every two days to prevent acidification (Collignon *et al.* 1997; Torricelli *et al.* 2000).

3.2.1.4 Growth curve for osteoblasts

The growth curve was constructed in order that the production of ALP and osteocalcin could be normalised to cell number, thereby reducing density-variation errors caused by different cell growth rates. Three cell lines (n=3 animals) were cultured to passage 3 using standard tissue culture techniques previously described. The MSCs were trypsinised into suspension and, as described above, seeded into individual T225 flasks for analysis on days 1, 7, 14, 21 and 28. The cultures were maintained at 37°C with 5% CO_2 in the original growth media. An equal number of flasks were treated with OM. Cells were trypsinised from the flasks and counted on respective days using standard tissue culture techniques described in Chapter 2.2.1.3. The growth curve on osteogenically-induced MSCs also allowed observations of cell differentiation stages to be made – indicating when cells reached a secretory osteoblastic phenotype. In so doing, it indicated at what stage the impaction forces need to be applied to test the hypothesis. Sheep dermal fibroblasts (SDFs) were also treated with OM and photographed at days 7, 14, 21 and 28 as a negative control.

3.2.2 Characterisation of osteoblasts

3.2.2.1 ALP assay

To confirm osteoblast differentiation, markers to identify cells as osteoblasts are a prerequisite. The basic osteoblastic phenotype is characterized by Alkaline Phosphatase activity, osteocalcin production and mineralization of the extracellular matrix analysed by Von Kossa staining. Assays for the proteins and mineralisation were carried out on 3 different cell lines at defined time-points during the

differentiation process – days 1, 7, 14, 21 and 28. ALP and OC assays were carried out on non-osteogenically induced MSCs (n=3 sheep) which acted as negative controls. Negative controls for the Von Kossa stain were SDF cells treated with osteogenic media.

At the end of days 1, 7, 14, 21 and 28 the following procedure was carried out for the biochemical assay. The media in the flasks was discarded and replaced with 4ml of distilled water. The flask was placed in the -70°C freezer to lyse the cells and then thawed in a 37°C incubator twice. The samples were thawed at 4°C overnight to allow regeneration of enzyme activity. ALP activity was measured in 3 x 50µl samples of each cell lysate from each cell line. Samples were assayed by an automated process in the COBAS BIO centrifugal analyzer (Roche,UK). The reagent p-nitrophenol phosphate (RANDOX Laboratories Ltd, UK) was added to the samples, whereby ALP cleaved the phosphate group to give p-nitrophenol which is yellow at alkaline pH and is monitored in the COBAS BIO (Roche, UK) at 405nm. This gave a measurement of the activity of the enzyme.

3.2.2.2 Osteocalcin assay

The osteocalcin assay was performed on the cell lysate extracted according to the protocol described in section 3.2.2.1 above. Osteocalcin was measured in 2 x 25µl samples of each cell lysate from each cell line using an Osteocalcin kit according to the protocol provided by the manufacturer (Metra-Biosystems OC Enzyme immunoassay). The optical densities of samples were measured at a wavelength of 405nm using a Dynatech MR700 plate-reader (Dynatech Laboratories, UK).

3.2.2.3 Histochemistry of mineralized matrix formation

Von Kossa staining was used to detect phosphate deposition, which is representative of the formation of bone nodules; allowing an assessment of matrix mineralization to be made – another indicator of osteoblastic differentiation (Holy *et al.* 2000; Erices *et al.* 2000). The stain was performed on the same days as the assays. Fresh stocks of 2% Silver nitrate solution (Sigma S2252), 2.5% Sodium thiosulphate (BDH Chemicals 10268) and 1% Neutral red (Sigma N6634) were

freshly prepared in distilled water on the day of the experiment. The incubating medium was discarded and cells were fixed in ice-cold 80% methanol. Sections were taken to water, then placed under a bright light in the presence of a 2% Silver nitrate solution for 1 hour. They were then rinsed 3 times with distilled water, treated with 2.5% Sodium Thiosulphate for 2 minutes and then rinsed in tap water. The Neutral red solution was filtered prior to use, and then used to counterstain the section for 10 minutes. Sections were rinsed several times to remove residual dye and left to dry. Sections were examined using a light microscope. Digital Photos were taken using a x40 objective.

3.2.3 Preparation of allograft

Ovine long bones were harvested, morsellised and sieved to 2-5mm in diameter. The allograft was double bagged with an airtight heat seal into 3.5g aliquots and irradiated and stored as described in the Materials and Methods section of Chapter 2, section 2.2.2.

3.2.4 Determining impaction forces

Impaction forces were established in Chapter 2 section 2.2.3.2 and based on the work of Dr Kirsty Phipps (Phipps *et al.* 2002). The average forces that allograft received during revision THR were in a range between 3kN and 6kN with the highest forces reaching 9kN (Phipps *et al.* 2002). These forces were replicated in this study using the specially designed impactor shown in figure 2.2.

3.2.5 *In vitro* impaction of osteoblasts on allograft

3.2.5.1 Suspension seeding of osteoblasts onto allograft

Sheep MSCs treated with osteogenic media for 11 days, to allow for differentiation down the osteogenic pathway, were trypsinised from tissue culture flasks. All cells were subconfluent at passage 4. After this point the seeding technique was similar to that used in Chapter 2, section 2.2.4. Osteoblasts were counted and seeded at 2 x 10^6 cells/g onto 3.5g of allograft in a universal tube with 7ml DMEM, 10% FCS and penicillin / streptomycin (1000iu/ml; 100µg/ml). A control universal tube contained allograft only with 7ml of DMEM. Cells were suspension seeded on the

allograft by rotation on a TAAB rotator (TAAB, UK) for 2.5 hours at 37°C with 5% CO_2. The constructs were then transferred to a 6 well plate and were maintained in culture for 72 hours pre-impaction, to allow attachment of cells to the graft. There were 6 samples in each force group, including the 0kN control group where osteoblasts were added but no impaction force was used. In addition, a small portion of the sample was removed for SEM analysis after 72 hours in culture to ensure attachment of cells to the graft.

3.2.5.2 Impacting constructs in delrin tubes

The impaction procedure was a replica of the protocol in section 2.2.5.2 using osteoblasts derived from MSCs.

3.2.5.3 Alamar Blue™ Assay

The Alamar Blue™ assay followed the same procedure as in section 2.2.5.3.

3.2.5.4 SEM Analysis

SEM analysis followed the same procedure as in section 2.2.5.4.

3.2.6 Statistical analysis

Statistical analysis for cell metaboliosm in Alamar Blue™ was carried out in the same way as in section 2.2.6.

3.3 RESULTS

3.3.1. Characterisation of osteoblasts

3.3.1.1 Observations of MSC differentiation in vitro

The images in figure 3.1 demonstrate the morphological changes occurring in adherent MSCs at specified time intervals during 28 days of differentiation compared to the SDF control. At day 7 MSCs had a fibroblastic spindle-shaped morphology to SDFs. By day 14, the MSCs had become progressively more cuboidal and polygonal. However, control cultures maintained a bipolar shape throughout the assay period becoming progressively more confluent. Osteogenically induced cells then became increasingly round in appearance and despite initial proliferation osteoblasts were more sparsely distributed by day 21.

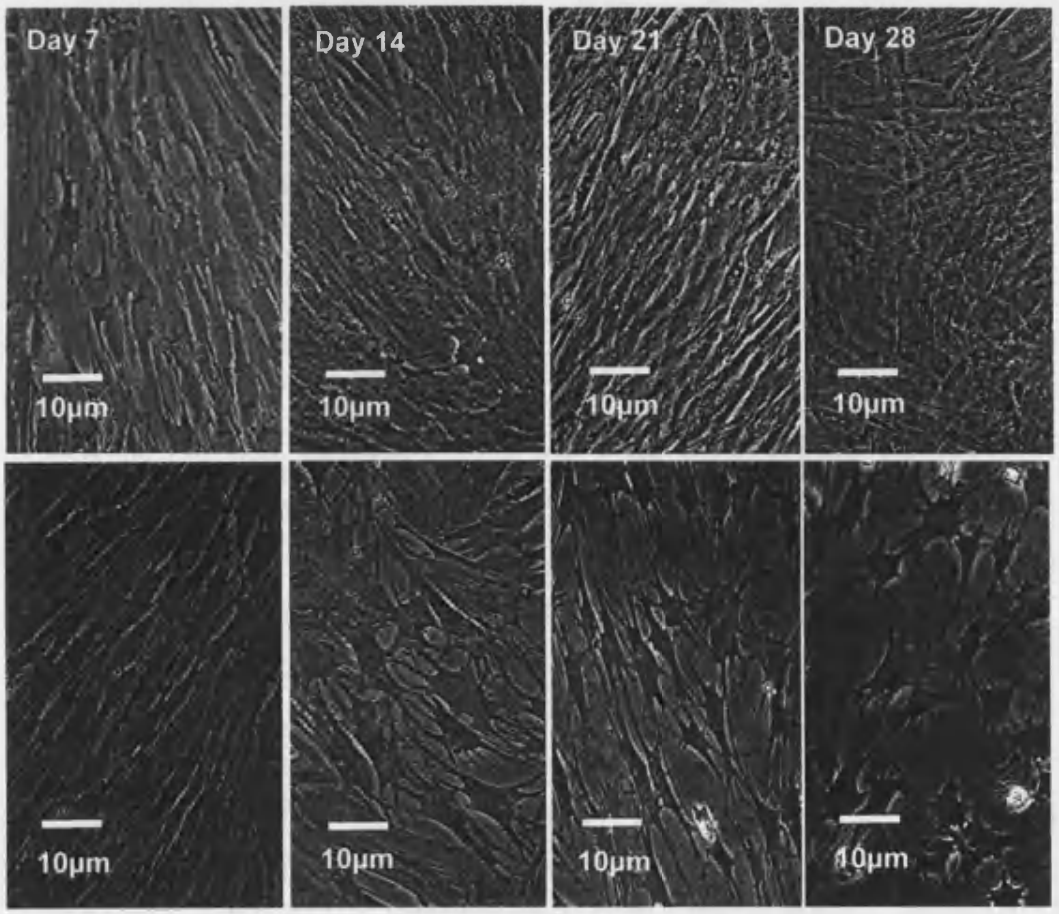


Figure 3.1 Phase contrast photographs of cells during assay period. Top row: SDF (Control). Bottom row: osteogenically induced MSCs.

3.3.1.2 Growth curve for osteoblasts

Figure 3.2 shows the growth rate of the MSCs and MSCs treated with osteogenic media over a culture period of 28 days. A plateau in growth was observed after day 21 for control cells in regular media. MSCs treated with osteogenic media plateau in growth earlier and at a lower number of cells. Cells were seeded onto the allograft at day 11 because they were still in the growth phase, allowing 72 hours for attachment, continued maturity and differentiation of the cells on the allograft before impaction. It was also the same amount of time given to the MSCs to attach to the allograft before impaction in Chapter 2 section 2.3.1.2

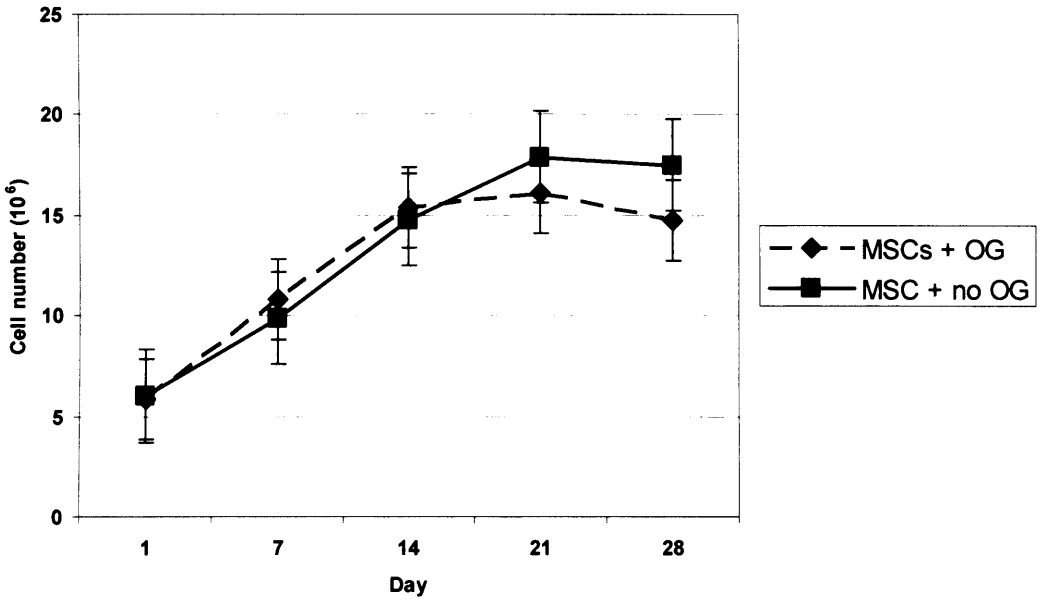


Figure 3.2 Graph to show the number of live MSCs in monolayer, treated with (MSC+OG) and without osteogenic media (MSC+no OG). Each MSC point (mean ± Standard Error) was derived from triplicate cultures.

3.3.1.3 ALP assay

Figure 3.3 shows the results for the ALP assay. At 1, 7 and 14 days the enzyme activity was always greater in the MSCs treated with osteogenic media than the control treated with standard media. The pattern of production of ALP in the MSCs treated with osteogenic media showed a significant increase between day 1 and day 7 ($p=0.05$) followed by a decrease at day 21 and at subsequent time-points during the assay. By day 7 ALP activity was significantly greater in the MSCs treated with osteogenic media compared to the control samples ($p=0.05$). The control MSCs that had not been treated with osteogenic media produced relatively low levels of ALP that did not vary significantly between time-points.

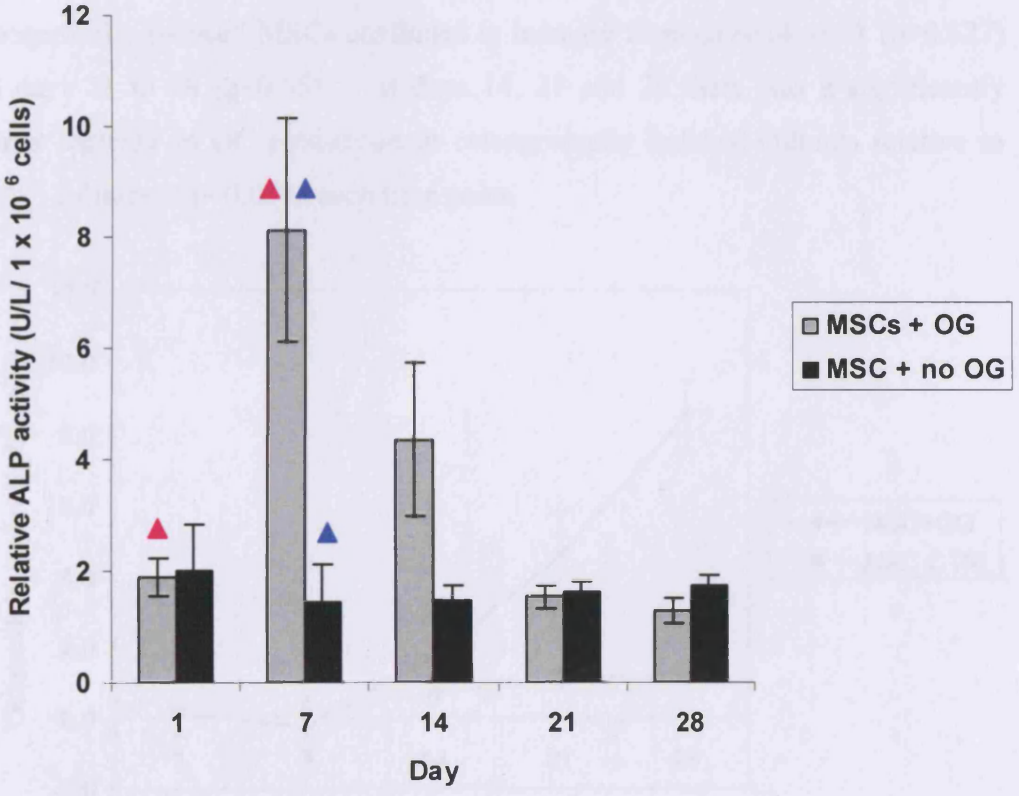


Figure 3.3 The relative induction of ALP activity in cells treated with and without osteogenic media at 7 day intervals for 28 days. Each point (mean ± Standard error) was derived from triplicate cultures. The Mann-Whitney U Test is represented by ▲ $p=0.05$, and ▲ $p=0.05$.

3.3.1.4 Osteocalcin assay

Figure 3.4 shows the results for the osteocalcin assay. Osteocalcin levels increased over a 28 day assay period in osteogenically treated MSC samples. At days 1 and 7 no osteocalcin was observed. However, the reading on day 7 shows negative osteocalcin production. This may be due to variations in the sensitivity of the Dynatech MR700 plate-reader (Dynatech Laboratories, UK). Osteocalcin levels significantly increased in the osteogenically induced MSCs ($p=0.05$) between days 7 and 14. A smaller increase in osteocalcin was observed in control MSCs between days 7 and 14, but this was not significant ($p=0.127$). Osteocalcin production in control MSCs increased slightly between days 14 and 21 ($p=0.275$) and plateaued between days 21 and 28 ($p=0.827$), although there was significantly more osteocalcin on day 28 than on day 14 ($p=0.05$). Osteocalcin production in osteogenically induced MSCs continued to increase from days 14 to 21 ($p=0.127$) and days 21 to 28 ($p=0.05$). At days 14, 21 and 28 there was a significantly greater increase in OC production in osteogenically induced cultures relative to control cultures at $p=0.05$ at each time point.

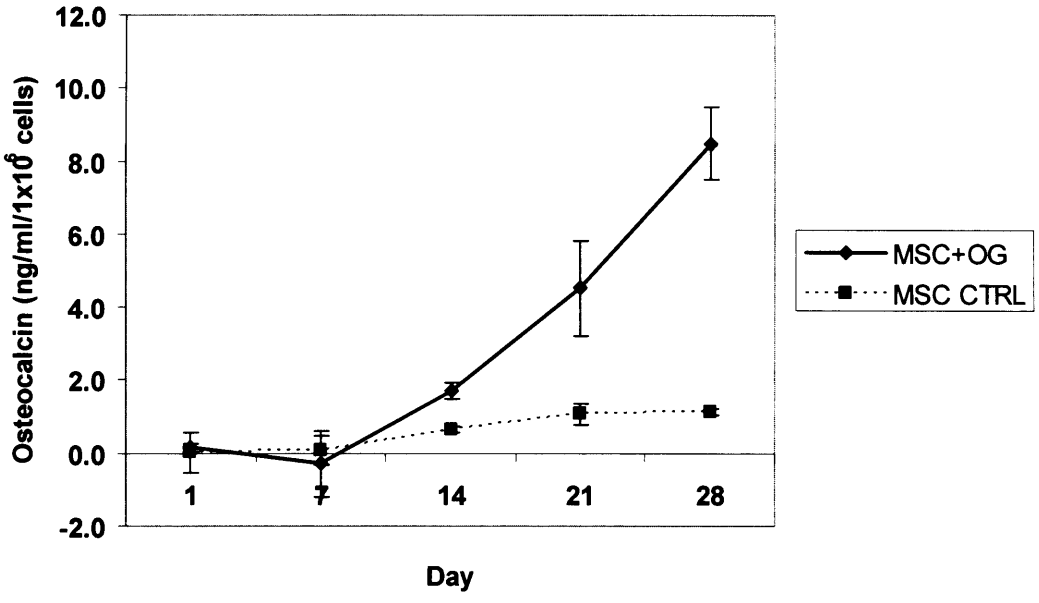


Figure 3.4 The relative production of osteocalcin by cells treated with and without osteogenic media over 28 days (mean ± Standard Error) (MSC CTRL= mesenchymal stromal cells control; OG = Osteogenic Media).

3.3.1.5 Histochemistry of mineralised matrix formation

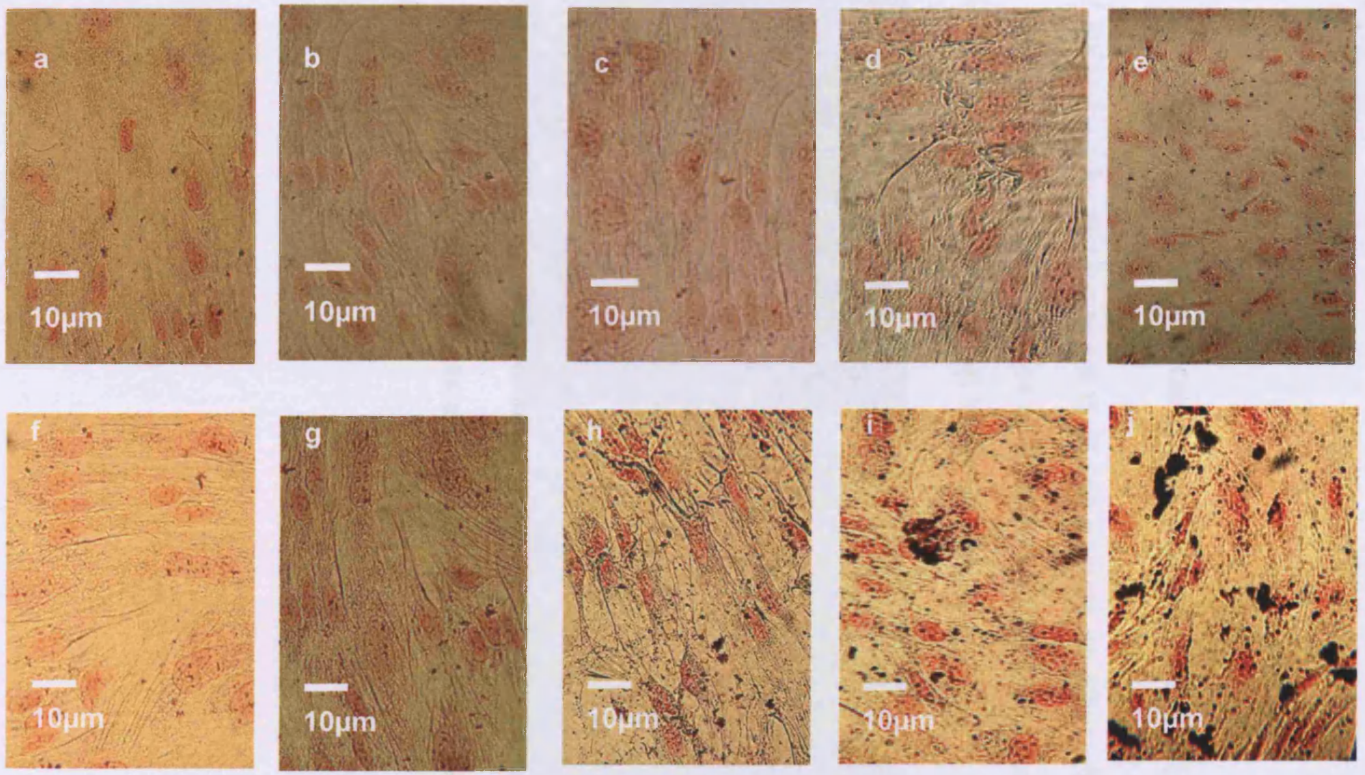


Figure 3.5 Von Kossa stain for mineralisation; Top row **a-e)** SDF negative controls at days: **a)** 1, **b)** 7, **c)** 14, **d)** 21, **e)** 28; Bottom row **f-j)** MSCs treated with osteogenic media on days: **f)** 1, **g)** 7, **h)** 14, **i)** 21, **j)** 28.

Figure 3.5 shows SDF control cells treated with standard media and osteogenically treated MSCs. The osteogenically treated MSCs show increasing mineral deposition between 14 and 28 days. The SDF controls show no mineral deposition at any timepoint.

3.3.2 *In vitro* impaction of osteoblasts on allograft

3.3.2.1 SEM for suspension seeding of osteoblasts on allograft

Figure 3.6 below shows SEM images of a network of rounded osteoblasts on the surface of the allograft on the day just before impaction.

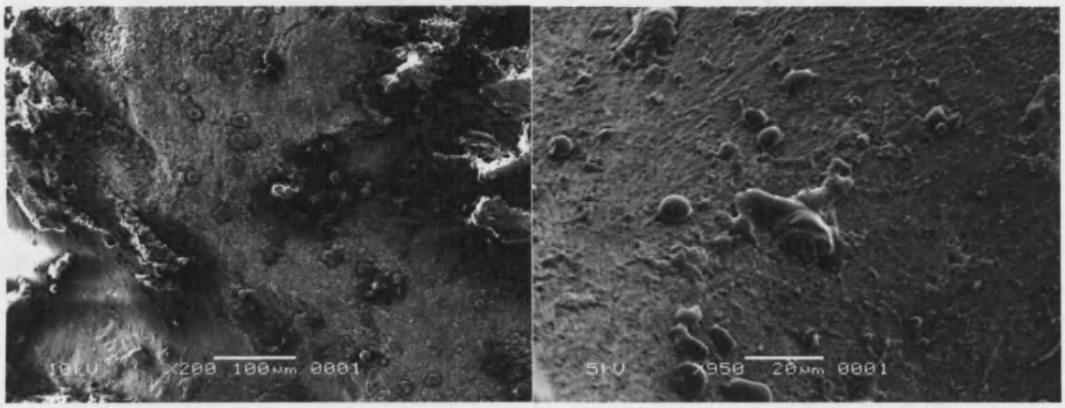


Figure 3.6 SEM showing rounded cells attached to the allograft surface on the day of impaction 3 days post-seeding.

3.3.2.2 Alamar Blue™ results

Figure 3.7 below shows the effect of impaction on osteoblasts at a range of normal forces used during THR surgery, where metabolism of the cells was assayed using Alamar Blue™. The red line on the graph depicts the original, pre-impaction level of metabolism of the osteoblasts at 100% and is a marker for recovery of the cells. The metabolism of the osteoblasts drops below this level in all groups after impaction. Compared with the 0kN control on day 2 (the first day post-impaction), the impaction at 3kN slightly decreased metabolism but showed no significant difference ($p=0.110$). However, the metabolism of osteoblasts impacted at 6 and 9kN was significantly less than the control at day 2 ($p<0.001$ in both cases).

By day 5 and day 8 after impaction, osteoblasts in the 3kN group recovered above levels of original pre-impaction metabolism, indicating recovery. Although there was an increase in metabolism over the 8 day period post-impaction, the differences in metabolism of the 3kN group relative to the 0kN control gained significance from day 5 at $p=0.025$ to day 8 at $p=0.020$. However, there was a decrease in metabolism by day 1 in the 6kN and 9kN groups with a further decrease by day 9. The difference in metabolic activity remained highly significant relative to the 0kN control for both the 6 and 9kN groups ($p<0.001$).

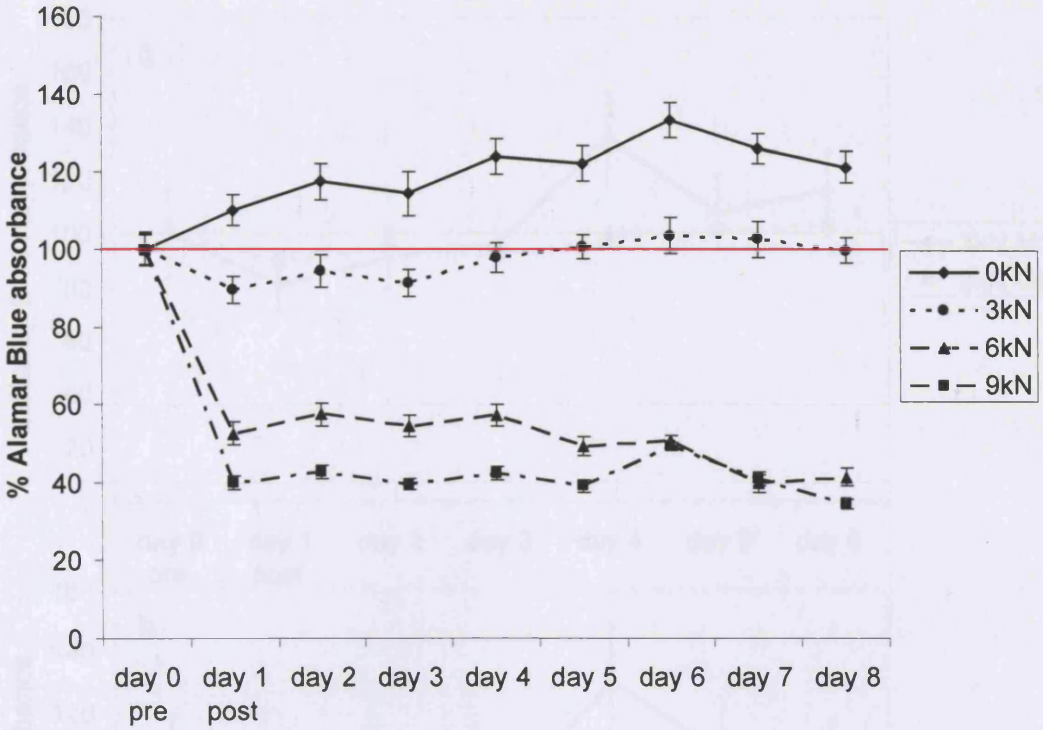


Figure 3.7 Percentage absorbance of Alamar Blue™ on cells in impacted and non-impacted graft. A red line marks 100% metabolism of the OB cells and represents the original metabolism of the OB cells before impaction.

The graphs in figure 3.8 directly compare the effects of impaction on MSC metabolism, analysed in Chapter 2, and osteoblast metabolism. At 3kN both cell types show a similar trend in cell metabolism. Post-impaction, on day 1, both MSCs and osteoblasts incur a drop in metabolism followed by recovery to levels above the pre-impaction value (figure 3.8a). However, in the 6kN group, although MSC metabolism recovers by day 4 and continues above pre-impaction levels until day 6, osteoblast metabolism continues dropping after impaction. The trends in metabolism between the MSC and osteoblast group are similar at 9kN impaction, with osteoblasts slightly more affected by impaction. Osteoblast metabolism does not recover after impaction.

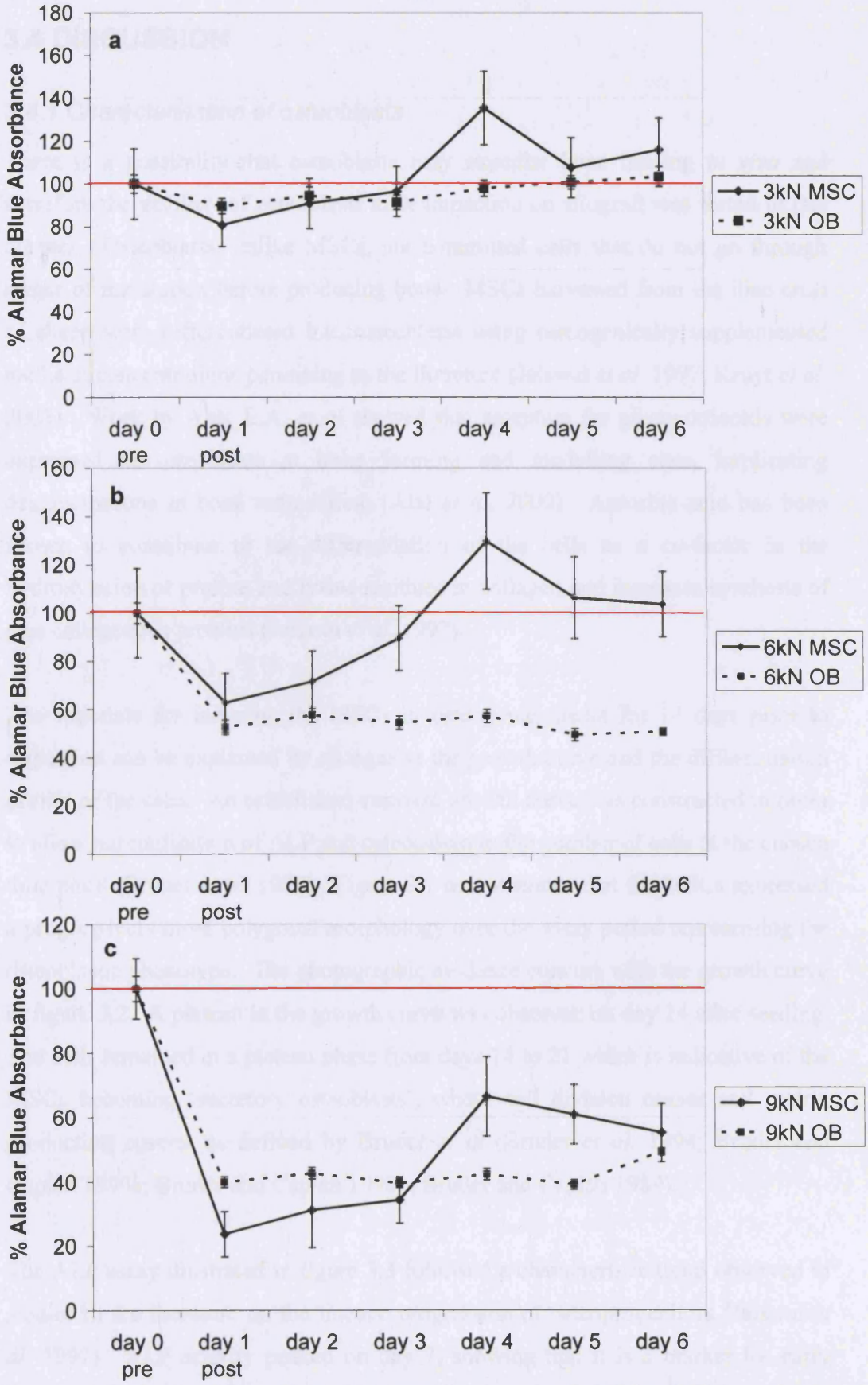


Figure 3.8 Comparison of percentage absorbance of MSCs (MSC: n=9) to osteoblasts (n=6) at **a** 3kN, **b** 6kN and **c** 9kN impaction.

3.4 DISCUSSION

3.4.1 Characterisation of osteoblasts

There is a possibility that osteoblasts may expedite bone healing *in vivo* and therefore the viability of osteoblasts after impaction on allograft was tested in this chapter. Osteoblasts, unlike MSCs, are committed cells that do not go through stages of maturation before producing bone. MSCs harvested from the iliac crest of sheep were differentiated into osteoblasts using osteogenically supplemented media at concentrations pertaining to the literature (Jaiswal *et al.* 1997; Kruyt *et al.* 2003). Work by Abu, E.A. *et al* showed that receptors for glucocorticoids were expressed in osteoblasts at bone forming and modelling sites, implicating dexamethasone in bone remodelling (Abu *et al.* 2000). Ascorbic acid has been shown to contribute to the differentiation of the cells as a co-factor in the hydroxylation of proline and lysine residues in collagen and increases synthesis of non-collagenous proteins (Jaiswal *et al.* 1997).

The rationale for inducing the MSCs in osteogenic media for 14 days prior to impaction can be explained by changes in the growth curve and the differentiation profile of the cells. An established standard growth curve was constructed in order to allow normailisation of ALP and osteocalcin to the number of cells at the chosen time point (Bruder *et al.* 1997). Figure 3.1 demonstrates that the MSCs expressed a progressively more polygonal morphology over the assay period representing the osteoblastic phenotype. The photographic evidence concurs with the growth curve in figure 3.2. A plateau in the growth curve was observed on day 14 after seeding. The cells remained in a plateau phase from days 14 to 21 which is indicative of the MSCs becoming 'secretory osteoblasts', where cell division ceases and matrix production ensues, as defined by Bruder *et al* (Bruder *et al.* 1994; Bruder and Caplan 1990a; Bruder and Caplan 1990b; Bruder and Caplan 1989).

The ALP assay illustrated in figure 3.3 followed a characteristic trend observed in studies in the literature on the lineage progression of osteoprogenitors (Jaiswal *et al.* 1997). ALP activity peaked on day 7, showing that it is a marker for early osteogenesis, and was followed by an immediate decline in activity. The reduction in ALP activity correlates with increased mineralisation and upregulated

osteocalcin expression (figure 3.3). ALP is an enzyme that has already been described in Chapter 1 as prompting phosphate ions to accumulate on collagen fibrils (Young and Heath 2000) and is expressed in osteoblasts. ALP peaks in expression and then declines (Bruder *et al.* 1997) because the function of ALP is to encourage mineral deposition which requires collagen to be present first. Osteocalcin is the most abundant non-collagenous protein in bone and may have a role in bone remodelling (Beresford *et al.* 1984). Osteocalcin production is unique to bone and almost exclusive to osteoblasts (Hughes and Aubin 1998; Beresford *et al.* 1984). The growth profile, together with the ALP and osteocalcin data, established that the cells were sufficiently differentiated along the osteogenic lineage to warrant being termed osteoblasts. The techniques established that on and after day 14, the cultures contained a population of functionally active secretory osteoblasts.

3.4.2 Effect of impaction forces on osteoblasts

The hypothesis was found to be false that: **Osteoblasts derived from MSCs seeded onto allograft scaffolds will survive normal impaction forces used during rTHRs.** The Alamar Blue™ assay demonstrated that cell metabolism was significantly reduced after impaction at 6 and 9kN and did not recover above the pre-impaction level of metablolism, which is a marker for cell recovery. However, although there was a reduction in cell metabolism of the osteoblasts immediately post-impaction at 3kN, this was not significant. The osteoblasts in the 3kN group also recovered above original levels of metabolism after 96 hours post-impaction, indicating that the osteoblasts had either proliferated or recovered their biosynthetic activity following their initial shock (figure 3.7).

Figure 3.8 compares the changes in metabolism of MSCs from chapter 2, and osteoblasts from this chapter after impaction. Figure 3.8a and figure 3.8c shows there is not much contrast between the MSCs and osteoblasts in terms of metabolic levels after impaction at 3kN or 9kN. The most dramatic difference between the two cell types is at 6kN post-impaction. Initially both cell types incur a reduction in metabolic levels 24 hours post-impaction. MSC metabolism is reduced by about 38% and OB metabolism is reduced by approximately 50%. However the MSCs recovered after 96 hours, with metabolism exceeding pre-impaction levels by

approximately 30%. In contrast, metabolism of the osteoblasts continued to decrease after 24 hours post-impaction and within 96 hours was approximately half of their pre-impaction levels. The contrasting behaviour of these two cell types indicates that they have different phenotypic responses to mechanostimultaiton.

The mechanism for the relative susceptibility of osteoblasts to mechanical forces may be explained by studies in the literature. In a study by Weyts, F.F.A. *et al*, immortalised human fetal osteoblasts were subjected to increasing levels of stretch at varying stages of differentiation. A multitude of responses were observed at different lineage points and with the age of the cells. For instance, younger osteoblastic cultures were induced into apoptosis by stretch, whereas older cultures were unaffected. An increase in proliferation was differentiation dependent and occurred when ALP levels were high on day 14. This tight regulation on apoptosis by osteoblasts may be particular to 'secretory osteoblasts' as non-differentiated 14 day cultures did not show the same proliferative response to stretch (Weyts *et al.* 2003). This corresponds to the patterns of differing responses to impaction of the osteoblasts and MSCs in the study in this chapter, the difference being that the level of strain applied in this chapter is not known and is likely to be multi-directional. In addition, during differentiation the cells in the Weyts, F.F.A *et al.* study produced extracellular matrix (ECM) which may have affected the cellular responses to mechanostimultion (Weyts *et al.* 2003). In the work by Mikuni-Takagaki, Y. *et al*, rat calvarial cells of different osteogenic lineages were subjected to stretching on Matrigel. The most responsive cells were populations of intermediaries, between mature osteoblasts and osteocyte-like cells. Mineralisation was accelerated by stretching as evidenced by a decline in alkaline phosphatase and an increase in osteocalcin production (Mikuni-Takagaki *et al.* 1996). Their data, suggesting that mechano-responses are lineage dependent, corresponds to that of Weyts, F.F.A. *et al* with the additional suggestion that differentiation of the cells is further progressed by the application of strain.

Alamar Blue™ is an assay for the metabolic activity of cells. Proliferation, as well as increased cell biosynthetic activity, can lead to increased metabolic activity within a cell population. Conversely, cell death or a reduction in biosynthetic activity leads to a reduction in metabolism. Therefore, it is not possible to

distinguish between the relative influence of either proliferation or biosynthetic activity using Alamar Blue™ alone. Tritiated thymidine incorporation could have been used as an assay for cell proliferation. Cells that synthesise DNA incorporate the isotope and can be quantified using a scintillation counter. Another alternative is Hoescht 33258, which is a fluorescence dye for DNA estimation. The dye is specific for DNA and binds to adenine-thymine base pairs emitting fluorescence at a wavelength of 460nm. However this would not have been appropriate for my study as allograft contains native osteocytes and osteoblasts which would have contaminated the DNA reading (Freshney 2000). Both of the methods described above also require the removal of the cells from the allograft and the contents to be released from lysed cells. The cells may penetrate into pores of the graft so that the release of all of the cells from allograft by trypsinisation could not be guaranteed. Neither of these assays would have allowed continuous assessment of cells seeded on allograft over time, unlike Alamar Blue™.

3.5 CONCLUSION

Clinical impaction forces affect the viability of MSC derived osteoblasts seeded onto an allograft scaffold. However, osteoblasts can survive impaction forces up to 3kN, which is in the lower range of normal impaction forces used during revision THRs. Provided the force of impaction does not exceed 3kN, cells could potentially recover and reach their full osteogenic potential to produce bone *in vivo*. This could be possible through adaptation of the slap hammer, which would allow the force to be monitored so that it did not exceed 3kN during a revision THR (Phipps *et al.* 2002). The *in vivo* work that follows in this thesis will proceed with the use of both osteoblasts and MSCs. It may be possible to use osteoblasts for other clinical applications that do not require impactions such as spinal fusion, osteonecrosis of the femoral head and maxillofacial surgery.

CHAPTER 4: Bone remodelling of impacted allograft and hydroxyapatite with and without autologous MSCs

4.1 INTRODUCTION

Chapter two established that cells can survive impaction forces between 3KN and 6KN *in vitro.* It was essential to test the ability of tissue engineered constructs to induce bone formation in an orthotopic site in a large animal model after impaction at a force of 3kN. A large animal model is more relevant than a small animal model. The reasons are that a larger volume of graft material is required to represent the human clinical situation than is possible to test in a small animal model. Also, blood supply is associated with bone remodelling and the graft would be too close to vascularised surfaces in a small animal model. Therefore sheep were chosen for use in this Chapter and throughout this thesis.

A resemblance between the iliac crest of the sheep and human has been reported by Pastoureau *et al* (Pastoureau *et al.* 1989) in terms of access for biopsies. Sheep have been used to study musculoskeletal disorders such as: repair of fractures and articular ligaments, limb lengthening, treatment of osteoarthritis and osteoporosis. Other diseases studied include: muscular disorders, osteomyelitis, spinal diseases and biomaterial evaluation.

Healing tends to be faster in animals. The rate of bone repair is known to be inversely related to the position of the species on a phylogenetic scale (that is: evolutionary hierarchical scale). Therefore the rat has a higher capacity for bone regeneration than humans (Martini *et al.* 2004). In the Aerssens *et al.* study, the ash and IGF-1 content of trabecular bone in sheep was similar to humans. The main differences between sheep and humans was in the ash, hydroxyproline, extractable protein and IGF-1 content of cortical bone (Aerssens *et al.* 1997). Bone mineral composition does not differ between humans, cattle, sheep and dogs except in the early stages of growth. Cortical bone contains fewer Haversian canals in sheep than in human cortical, bone but the rate of bone healing in sheep is similar to the human healing rate. The femoral condyle was chosen because it contained the most cancellous bone. Before analysing the effect of impaction grafting with stem cells on hip replacements in an ovine model, it was desirable to assess bone formation using a more consistent, simple and controlled site. This model also allowed comparison between different grafts in the same model.

There is also literature pertaining to the efficacy of sheep as a large animal model for correction of osteolytic defects in revision THRs using mesenchymal stem cells. Tissue engineered constructs have been used successfully to heal critical sized defects in small (Kadiyala and Jaiswal 1997) as well as large animal models such as dogs and sheep (Kon *et al.* 2000; Bruder *et al.* 1998b). The critical sized defect is: 'the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of that animal' (Martini *et al.* 2004). In a study by Griffon *et al.* unicortical, metaphyseal defects of 15mm were created in ovine long bones and empty defects served as negative controls by having the lowest density after 7 weeks (Griffon *et al.* 2001). The diameter of the sheep defects in this Chapter was set at 15mm to prevent spontaneous healing within 6 weeks.

Aim

The objective of this Chapter is to assess whether new bone formation of impacted allograft and HA in a critical sized defect is improved by using autologous ovine MSCs *in vivo*. In addition osteoconductivity of irradiated allograft was compared with 60 percent porous granular HA in an animal model that simulates bone remodelling within impacted graft *in vivo*.

Hypotheses

1. **Addition of MSCs to allograft or HA used in impaction grafting will increase new bone formation *in vivo*.**
2. **Allograft will produce more new bone than HA because of its osteoinductive nature.**

4.2 MATERIALS AND METHODS

4.2.1 Study design

Twelve skeletally mature mule sheep were used to assess the effect of MSCs on new bone growth and remodelling in impacted graft. Six female sheep received morsellised allograft and six received HA granules. Defects of 15mm diameter were created in the medial femoral condyle on both right and left sides of the sheep. The graft was inserted at a quantity of 3.5g to both femoral condyles. In each sheep one condyle served as a control, containing graft alone, and the other condyle contained the same graft type with MSCs.

The groups were:

Sheep	Graft type	Femoral condyle: left or right	Femoral condyle: left or right
n=6 sheep	Allograft	control	MSC
n=6 sheep	HA (ApaPore-60™)*	control	MSC

*Apa Tech Ltd, Hertfordshire, UK

Table 4.1 Study design

The animals were euthanized at six weeks. The femoral condyles were removed for hard grade histology processing and histomorphometric analysis.

4.2.2 Preparation of graft

4.2.2.1 Allograft

Allograft was prepared as described in Chapter 2, section 2.2.2.

4.2.2.2 HA

The HA was provided in the form of ApaPore-60™ by Apa Tech (Apa Tech Ltd, Hertfordshire, UK). ApaPore-60 is a phase-pure synthetic HA with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. This material is used in clinical practice for impaction grafting. This was supplied as irregularly sized granules of 2-5mm in diameter

with a 60% porosity with an average pore size of 400μm. This HA has an interconnected structure. The HA was double bagged with an airtight heat seal into 3.5g aliquots and irradiated as described in the Materials and Methods section of Chapter 2.

4.2.3 MSC Culture

MSCs were harvested as bone marrow aspirates from the 12 sheep designated for the study. The cells used in the study were fibroblastic in appearance once adherent to tissue culture plastic and displayed this morphology throughout culture. The MSCs were expanded in culture until they were between passage 3 and 6 to achieve adequate numbers for cell seeding onto graft. Cell culture was done in accordance with the protocols described in chapter 3.

4.2.4 Suspension seeding MSCs onto allograft and HA

The same method for suspension seeding of graft was used as in Chapter 2. MSCs were removed from tissue culture plastic by trypsinisation and seeded at $2x10^6$ cells/g of allograft or HA. Cells were attached to the allograft by rotation on a TAAB rotator (TAAB, UK) for 2.5 hours at 37°C with 5% CO_2. The cells were then transferred to a 6 well plate and were maintained in culture for 72 hours pre-surgery. The graft was covered by 7ml of media.

4.2.5 Surgery

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 at the Royal Vetinary College (RVC), North Mymms, as described in Chapter 2. Twelve skeletally mature sheep were used for the study. At 48 hours prior to the operation the animals were housed separately and animals were starved for 12 hours pre-operatively.

4.2.5.1 Analgesia

At induction each animal was administered 0.6mg of Buprenorphine (2ml of 0.3mg/ml) intramuscularly for intraoperative analgesia. The dose was repeated on the first post operative day.

4.2.5.2 Impaction of the graft

Graft was placed into titanium tubes, having the same dimensions as the Delrin tubes used in Chapter 3. Titanium tubes were used for ease of sterilisation. Graft was added and the tube was then mounted into the impactor (figure 4.1a). In Chapter 2 MSCs were shown to have survived impaction forces of 3kN and 6kN. As described in Chapter 2 MSCs impacted at 3kN recovered faster than those impacted at 6kN and therefore a force of 3kN was chosen for this study. As in Chapter 2, section 2.3.2.3 twenty impactions were applied to the graft and MSC constructs as most graft compaction is achieved in the first twenty impactions (figure 4.1b). After HA samples were placed into the titanium tubes and before impaction, they were immediately covered with autologous blood from the jugular vein of the sheep which was allowed to coagulate before impaction. Addition of blood is normal clinical practice suggested by the manufacturer (Apa Tech, Hertfordshire, UK).

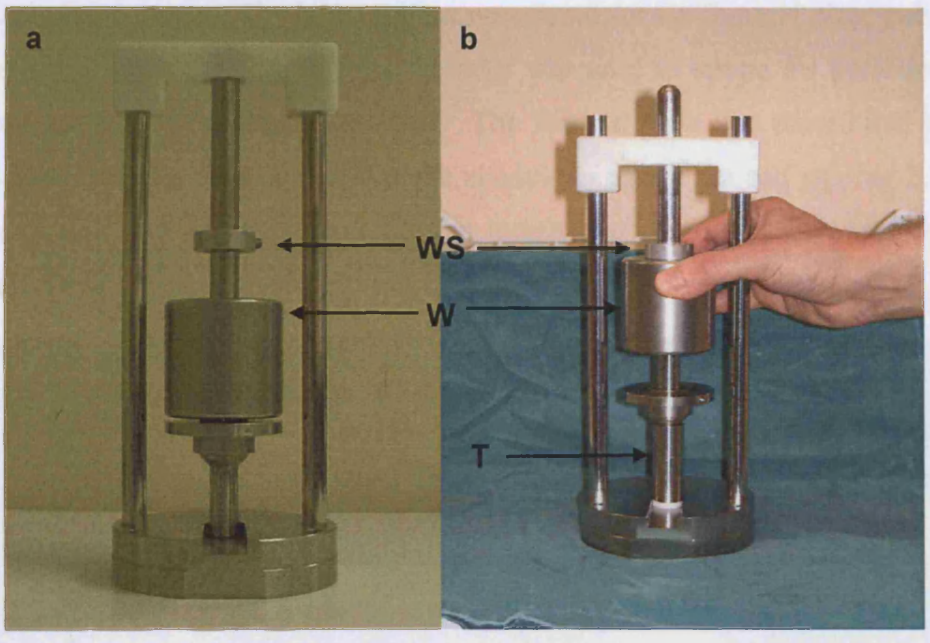


Figure 4.1a Illustrates the weight stop (WS) at 2.5cm above the weight (W); **b** shows that dropping the weight from this height delivers a force of 3kN to the graft contained in the titanium tube (T). Impactions were administered 20 times.

4.2.5.3 Insertion of graft

All surgical procedures were carried out under aseptic conditions. Once anaesthetised an area over the medial aspect of both knee joints, extending proximally over the medial thigh and lower abdomen, was shaved. Betadine (broad spectrum topical iodophor microbicide) surgical scrub was applied and the site was further cleaned with Hydrex Surgical Scrub (MidMeds Ltd, Loughton, UK), containing chlorohexidine, an alkaline aqueous antimicrobial (MidMeds Ltd, Loughton, UK).

Sterile drapes were used to expose the wound site whilst the animal was in the supine position for easy access to both left and right medial femoral condyles. A longitudinal 50 mm incision was placed over the medial aspect of the femur, just proximal to the knee joint and 30 mm posterior to the medial border of the patella. With sharp dissection of the superficial and deep fascia, the vastus medialis muscle was exposed. The muscle was then split along its fibers to expose the periosteum on the medial condyle. The best position was found for the femoral plate (which acts as a drill guide) and a periosteal elevator was used to scrape the periosteum from the surface of the femoral condyle. The femoral plate was placed and two 2mm diameter holes were drilled into the condyle to accept the self tapping 2.7 x 18mm cortical screws (Synthes, UK) that attach the femoral plate to the bone (figure 4.2).

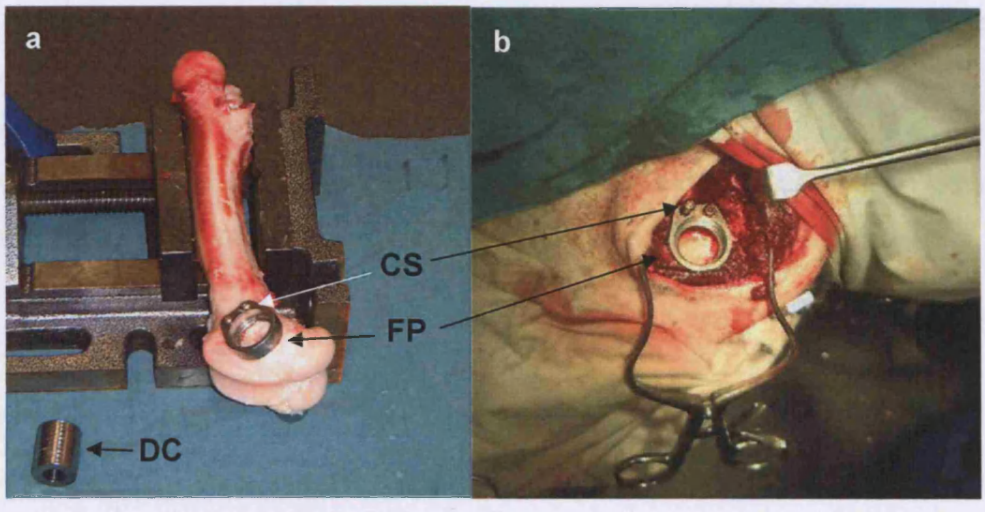


Figure 4.2a The image illustrates the femoral plate (FP) secured into place with cortical screws (CS) and a 10mm drill centraliser (DC) used to centralise the drill bit; **b** illustrates the FP in position *in vivo*.

The depth of the defect was determined by the height of the graft after impaction (figure 4.3a-b). The depth of the defect was drilled to 4mm deeper (Figure 4.3b) than the height of the pellet created by impacting the morselised allograft or granular HA to ensure that all the graft was contained within the defect and did not extrude from the top.

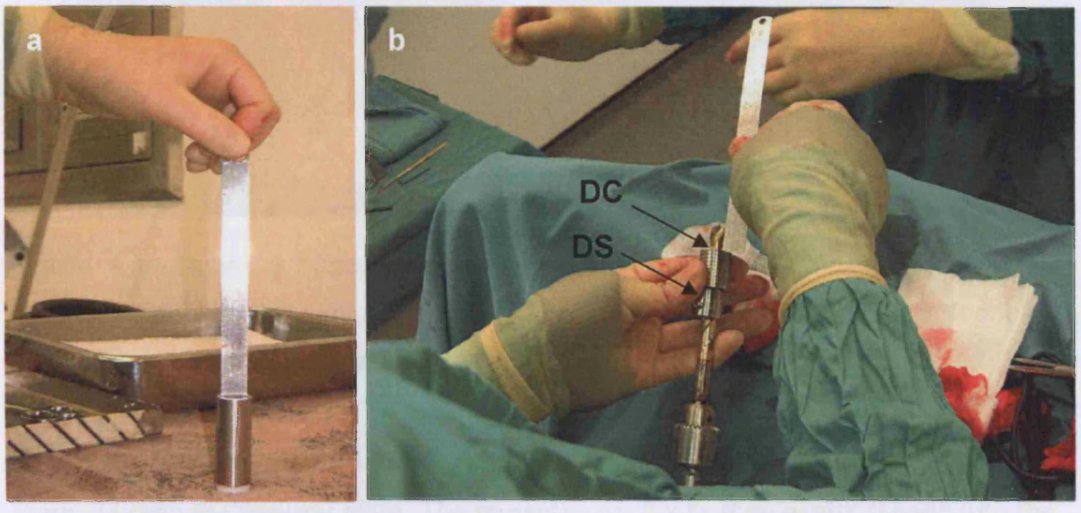


Figure 4.3a The graft height was measured using a sterile ruler. The measurement obtained was subtracted from the height of the tube (50mm). To give the final height another 4mm was subtracted as this was the depth that the delrin lid projected into the tube; **b** shows the depth of the defect plus the height of the DC being marked by the drill stop (DS).

Plugs were inserted transversely across the femoral bone. The cortex was reamed to produce a hole to accept a cylindrical shaped implant. 5, 10 and 15 mm pneumatic and hand held drills were used with the aid of drill centralisers, designed to enable accurate drilling of holes, to create the final 15mm diameter defect in the medial femoral condyle (Figure 4.4). The defect was washed using a syringe with sterile saline to remove bone debris.

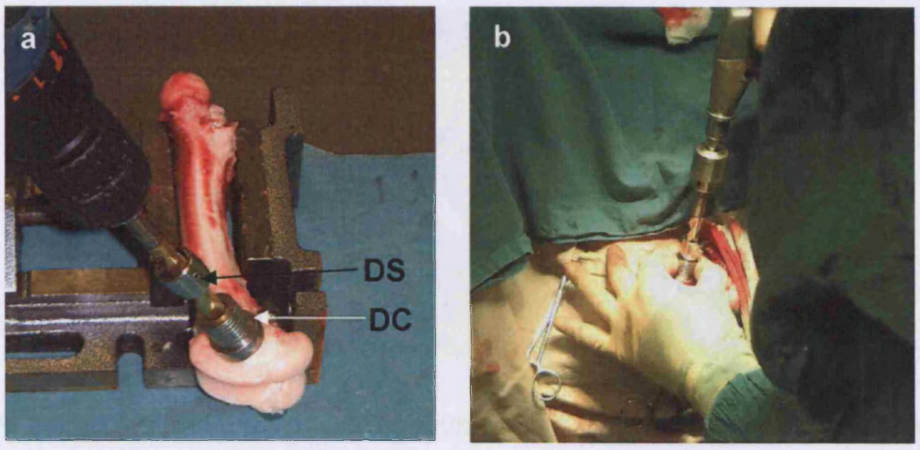


Figure 4.4a Shows a 10mm drill being guided through the DC mounted onto the femoral plate. The drill stop (DS) is placed on the drill bit to mark the exact depth for the drilling; **b** illustrates the drill creating a defect *in vivo*.

After impaction the delrin lid was removed and the tube containing the graft was mounted onto the femoral plate. The impacted graft was then gently tapped into the defect site through the femoral guide plate using a Delrin plunger and a mallet (figure 4.5). Once the graft had been driven in the femoral plate was then removed before wound closure.

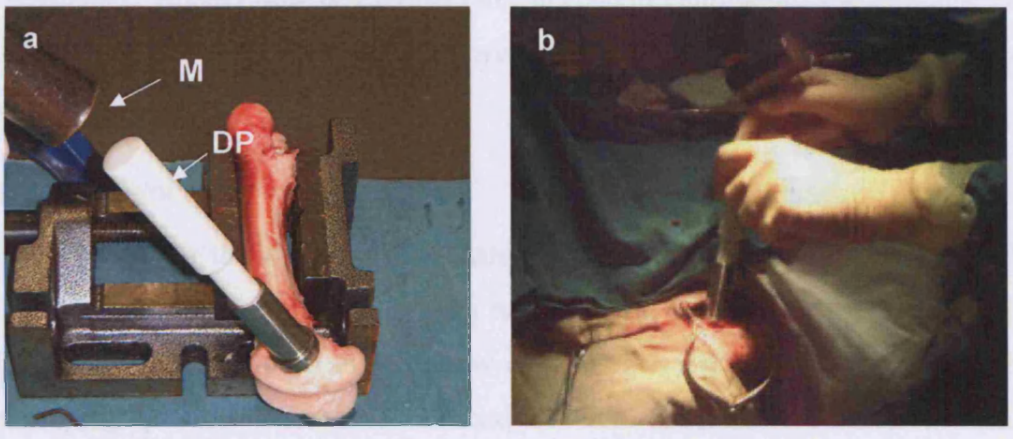


Figure 4.5a Shows the delrin plunger being used to tap the impacted graft and cell construct into the defect; **b** shows the placement of the graft using the same procedure *in vivo*.

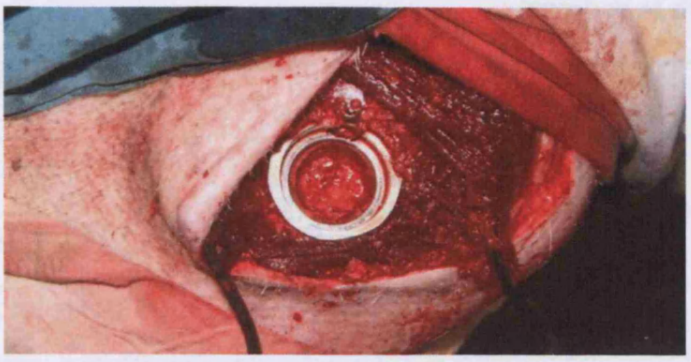


Figure 4.6 Shows the femoral plate surrounding the graft that has just been placed in the defect.

Once the plug was inserted the wound was closed in layers with absorbable vicryl™ sutures. The muscle apposed with 2 or 3 interrupted sutures, continuous closure of the deep fascia and dermis, with interrupted sutures to close the skin. Post-operatively the animals were recovered in sternal recumbency and housed in individual pens for 24 hours. A sample representing initial impaction for HA and allograft was impacted into the femoral condyles of anewly sacrificed sheep. The procedure outlined above was performed on the femur after the sheep had been euthanased. A daily dose of Ceforex (a cephalosporin antibiotic) was administered intramuscularly, for 5 days post operatively. The first dose was at induction of anaesthetic.

4.2.2.4 Histology

After six weeks the sheep were euthanized with Phenobarbitone (50ml of 20% solution) intravenously. Femurs were removed and debrided of soft tissue. The femurs were cut down on the bandsaw until the femoral condyles containing the graft plug remained. The femoral condyles were fixed in 10% buffered formal saline and dehydrated with solutions of ascending concentrations of IMS (BDH laboratory supplies, UK) in a similar manner to the protocol for resin embedding in chapter 2, section 2.2.5.4. However, as the samples were relatively large, a chloroform step was included for defatting to allow adequate penetration of the solutions prior to embedding in LR white resin (Agar Scientific Ltd, UK). The protocol for fixation is shown in table 4.1.

Process	Number of days
10% buffered formal saline	7
50% IMS 50% distilled water	3
70% IMS 30% distilled water	3
100% IMS (repeat step 3 times)	3
Chloroform (repeat Step twice)	1
100% IMS (change IMS twice per day)	3
50% LR white resin 50% IMS	7
100% LR white resin (under vacuum for 10 seconds) Apply vacuum every day	14
Cast in LR white resin (1 drop accelerator/10ml resin). Leave in fridge	24 hours

Table 4.2 Histology Processing Protocol

The samples were sectioned in the transverse plain using the Exact saw and ground to a thickness of 100μm using the Exakt micro-grinding system and polished on the Motopol 2000 (Bueler, Coventry, UK). Prior to histomorphometric analysis of the sections they were stained with Toludine Blue for 10 minutes, which stains fibrous tissue blue and a Paragon stain for 15 minutes which stains new bone bright pink.

4.2.6 Histomorphometry

4.2.6.1 New bone and remaining allograft

New bone and remaining allograft were quantified by area using the KS300 computer program. The samples were divided into three regions of interest: the middle, anterior host-graft interface and the posterior host-graft interface. The interface regions were selected 3mm away from the host bone and into the graft to

ensure no overlap with host tissue (figure 4.7) and to define reproducible regions that could be analysed.

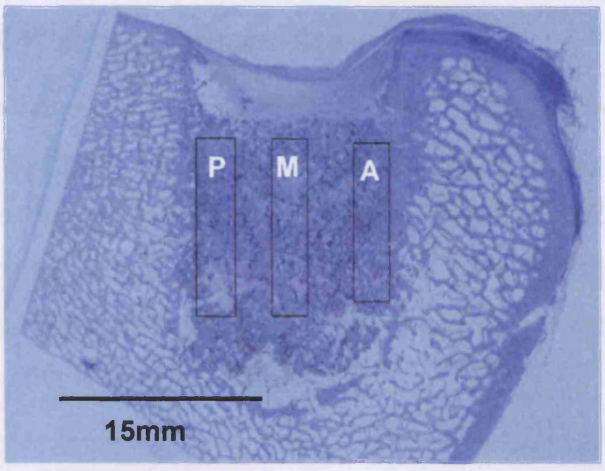


Figure 4.7 Image to show three regions of interest: Anterior host-graft interface (A), Middle (M) and Posterior host-graft interface (P).

Four pictures were taken at regular intervals along each region to give 12 images in total. Measurements were made of the field of view at x 4 magnification, the area of the remaining graft in the field of view and the area of new bone. Measurements for anterior and posterior interfaces were averaged to represent the host-graft interface. The data was analysed as percentage of remaining graft and percentage of new bone formation.

4.2.6.2 Porosity

Time zero samples were analysed to determine the porosity of the graft (the area occupied by the pores) and the graft area (expressed in %).

4.2.7 Statistics

The statistical software SPSS (SPSS v10.1, SPSS Inc, Chicago, USA) was used for the analysis. The Wilcoxon signed-rank test for non parametric data was used. Significance was established as $p \leq 0.05$. One sheep in the HA group had to be omitted from the data analysis due to extrusion of the graft from the control femoral condyle. Therefore 5 animals in the HA group were analysed rather than six. Bars on graphs represent standard error of the mean (±SE).

4.3 RESULTS

4.3.1 Allograft Histomorphometry

The mean for new bone and remaining allograft are presented in figures 4.8 and 4.9. The graph in figure 4.8 shows the differences in percentage new bone formation between the MSCs and the control within the interface and middle regions of the allograft group. The graph in figure 4.9 shows the differences in percentage of remaining graft between the control and MSCs within the interface and middle groups after six weeks *in vivo*.

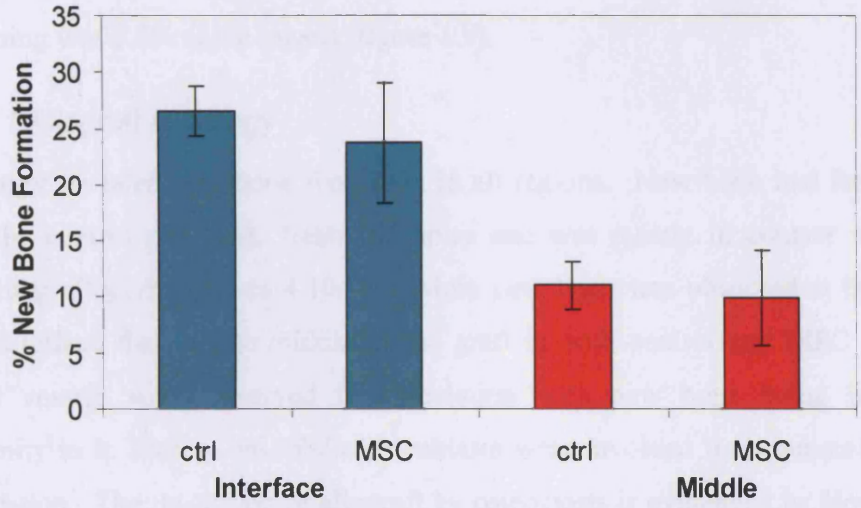


Figure 4.8 The graph represents percentage new bone formation at the middle and host-allograft interface (±SE) after six weeks *in vivo* after impaction at 3kN

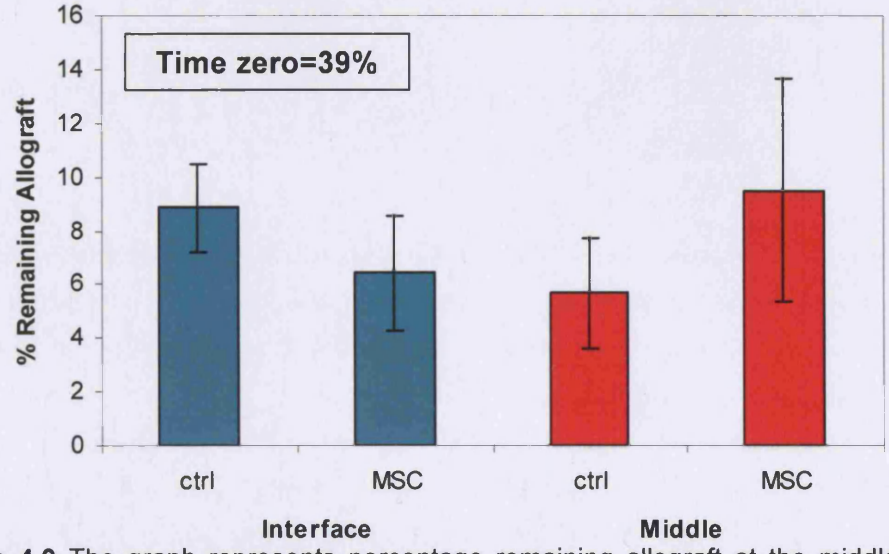


Figure 4.9 The graph represents percentage remaining allograft at the middle and interface (±SE) after six weeks *in vivo*. The amount of allograft occupying the microscopic field at time zero was 39%.

There was no significant difference in the mean amount of new bone formed in the MSC samples compared to the control samples at the interface with 23.6% ± 5.4 SE and 26.5% ± 2.1 SE (p=0.753) respectively, or the middle of the graft with 9.8% ± 4.2 SE and 11.0% ± 2.1 SE respectively (p=0.600). The area of allograft remaining was similar at both the interface in the control and MSC group at 8.9% ± 1.6 SE and 6.4% ± 2.1 (p=0.249) respectively and in the middle at 5.7% ± 2.0 and 9.5% ± 4.1 (p=0.500) in the control and MSC group respectively. After impaction at time zero the allograft had a porosity of 61%. The area of allograft occupying the microscopic field was 39%. After 6 weeks the area of allograft remaining was 5.6% at the lowest (figure 4.9).

4.3.1.1 Allograft Histology

Histology revealed new bone formation in all regions. New bone had formed in both the control and MSC treated samples and was mostly in contact with the remaining allograft (figures 4.10a-c). More new bone was observed at the host-graft interface than in the middle of the graft in both control and MSC groups. Blood vessels were observed in association with new bone being in close proximity to it. Plump, basophilic osteoblasts were involved in intramembranous ossification. The resorption of allograft by osteoclasts is evidenced by Howship's Lacunae, depressions in the bone surface seen in figure 4.10b.

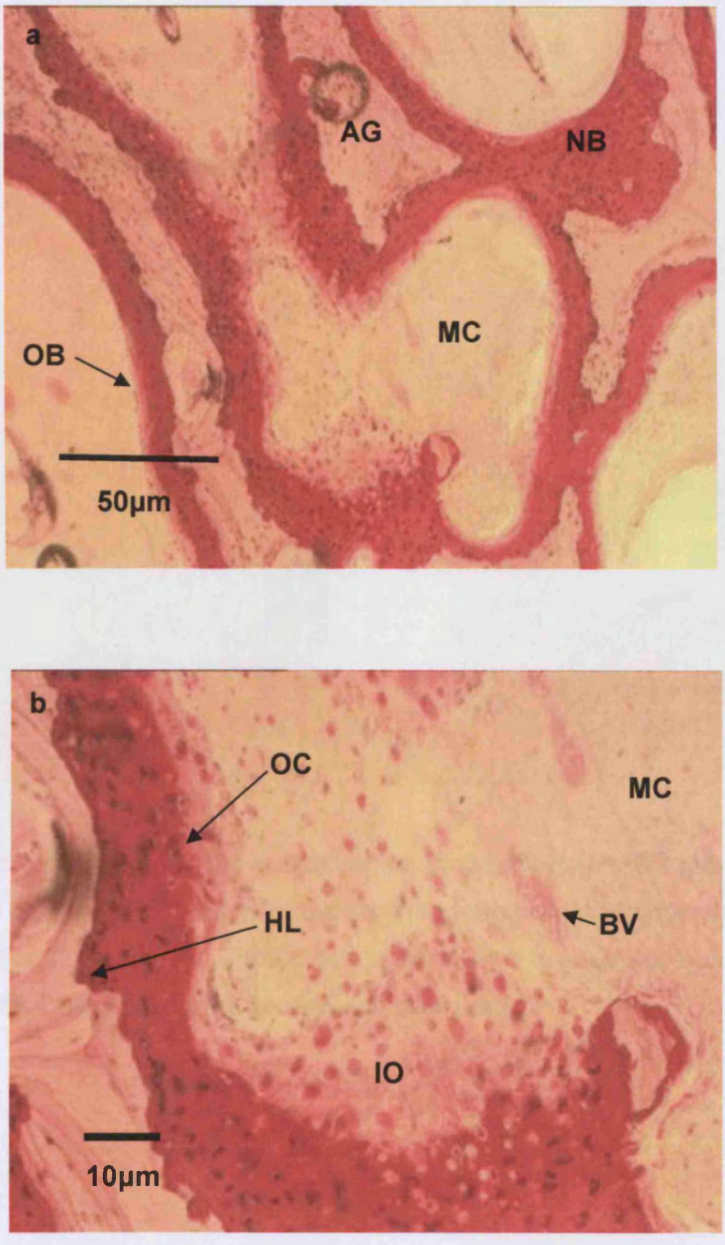


Figure 4.10a Image of the middle region in the allograft ctrl group. The paragon has stained new bone (NB) bright pink. The allograft (AG) remained light in colour. Active osteoblasts (OB) can be seen laying down osteoid, which stains lighter in colour on the surface of new bone. **b** During intramembranous ossification (IO) the osteoblasts become enclosed in the mineralising matrix becoming osteocytes. In this image osteocytes are relatively flattened, darkly stained specks. The Howship's Lacunae (HL) are depressions in the bone resorbed by osteoclasts that have preceded new bone formation. New bone is associated with angiogenesis; the marrow cavity (MC) contains blood vessels (BV).

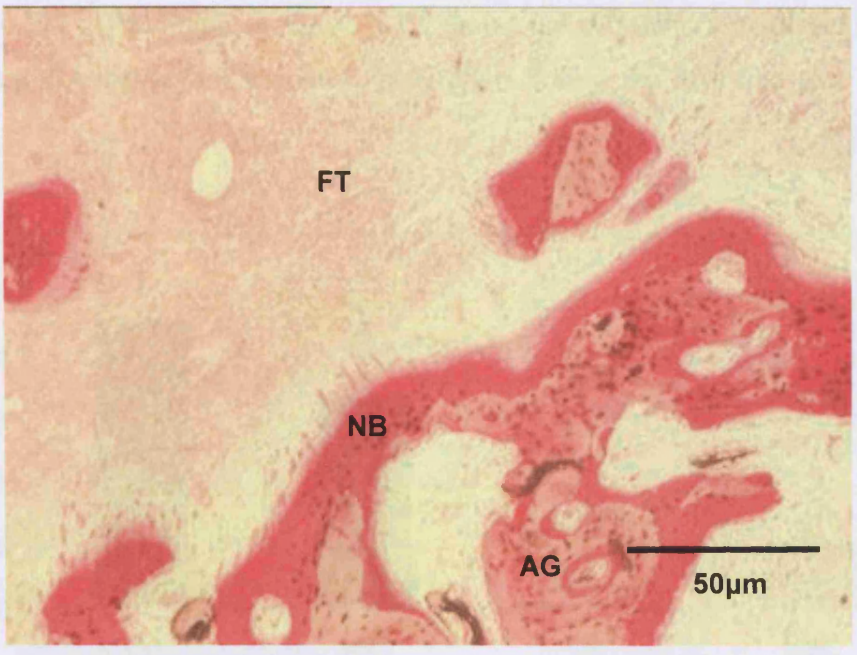


Figure 4.10c Image taken at the middle region of the allograft MSC group. The MSC group shows a similar morphology to the control group with NB forming in contact with remaining AG. There is also fibrous tissue filling all the gaps not occupied by new bone, allograft or other elements of mesenchymal tissue.

4.3.2 HA Histomorphometry

The graph below shows the differences in percentage new bone formation between the MSCs and the control within the interface and middle regions of the HA group (figure 4.11). The graph in figure 4.12 shows the differences in percentage of remaining HA between the control and MSCs within the interface and middle groups.

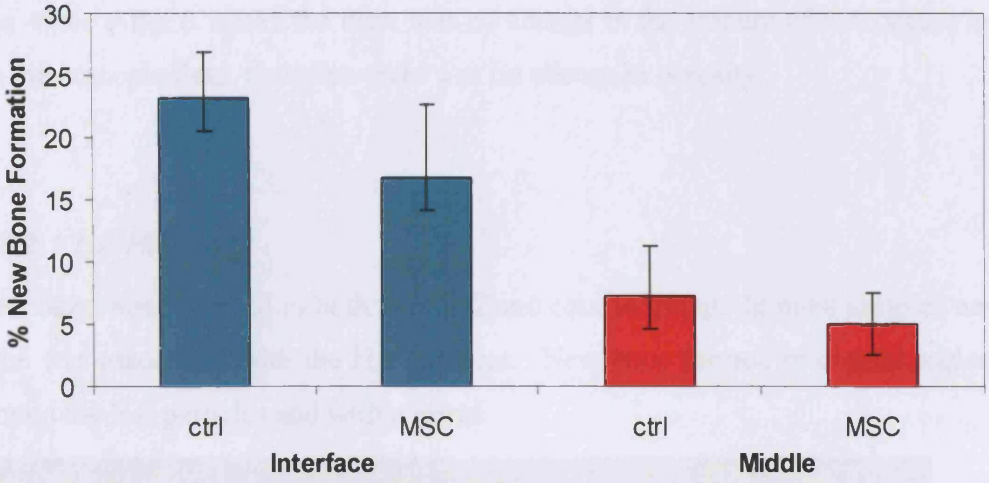


Figure 4.11 The graph represents percentage new bone formation at the middle and host-graft interface (±SE) after six weeks *in vivo* after impaction at 3kN

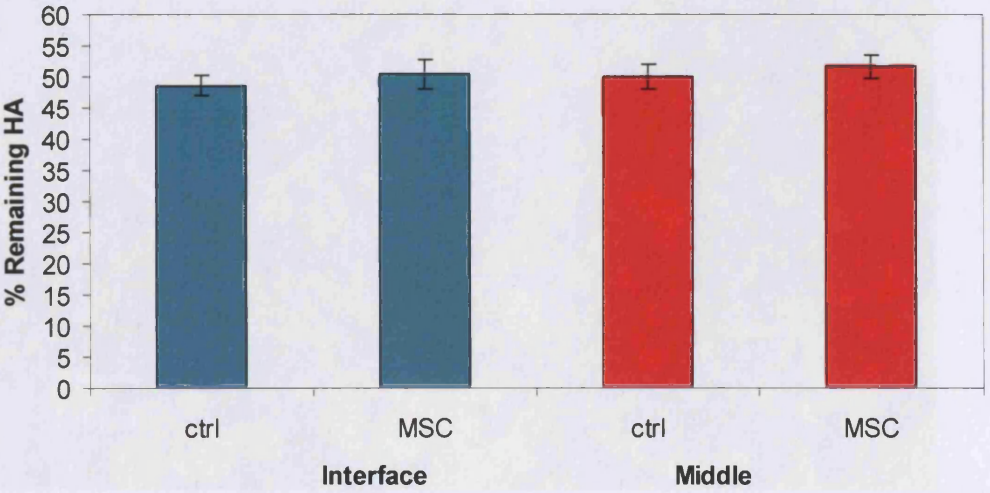


Figure 4.12 The graph represents percentage remaining allograft at the middle and interface (±SE) after six weeks *in vivo*. The area of HA occupying the microscopic field at time zero was 49%.

There was no significant difference in the mean amount of new bone formed in the MSC samples compared to the control samples at the interface, at 16.7% ± 5.9 and 23.1% ± 3.8 (p=0.800) respectively and the middle of the HA at 5.0 ± 2.6 and 7.2 ± 4.1 (p=0.273) respectively. The area of HA remaining was similar at both the interface in the control and MSC group at 48.6% ± 1.7 SE and 50.4% ± 2.4 (p=0.500) respectively and in the middle at 50.1% ± 2.0 and 51.7% ± 1.9 (p=0.500) in the control and MSC group respectively. After impaction at time zero the HA had a porosity of 51%. The area of HA occupying the microscopic field was 49%. After 6 weeks the there was no change in the amount of HA occupying the microscopic field, therefore there was no change in porosity.

4.3.2.1 HA Histology

New bone was observed in both the MSC and control group. In most samples new bone was associated with the HA particles. New bone formed in coastal regions around the HA particles and within pores.

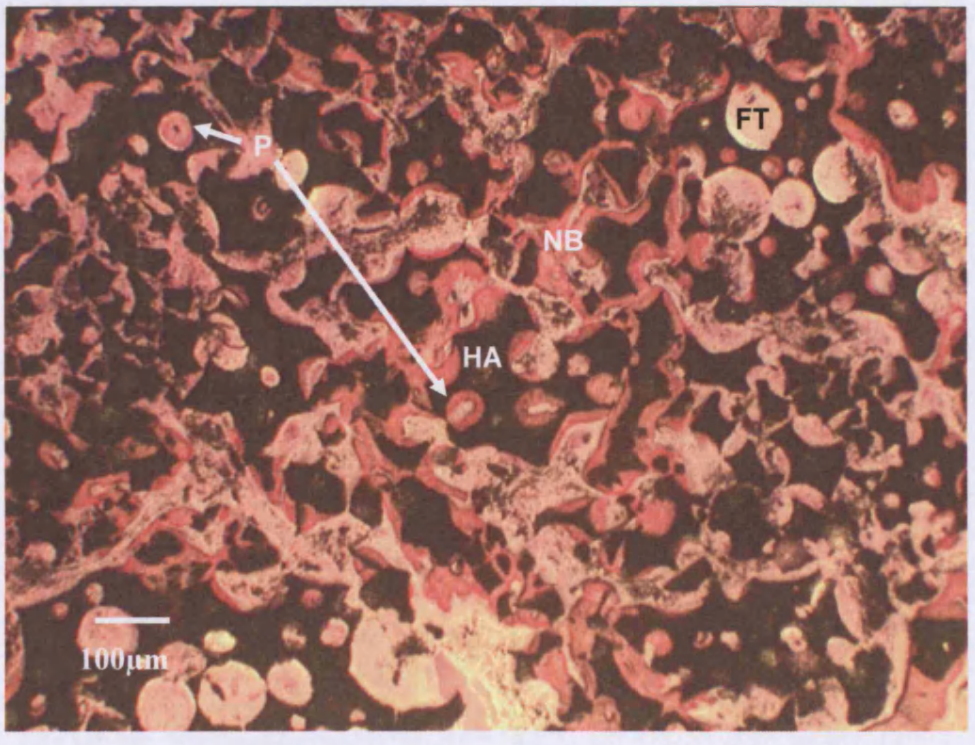


Figure 4.13 Image taken at the middle region in the HA control group after 6 weeks *in vivo*. Remaining HA (HA) appears black and contains pores (P) surrounded with new bone (NB) and fibrous tissue (FT).

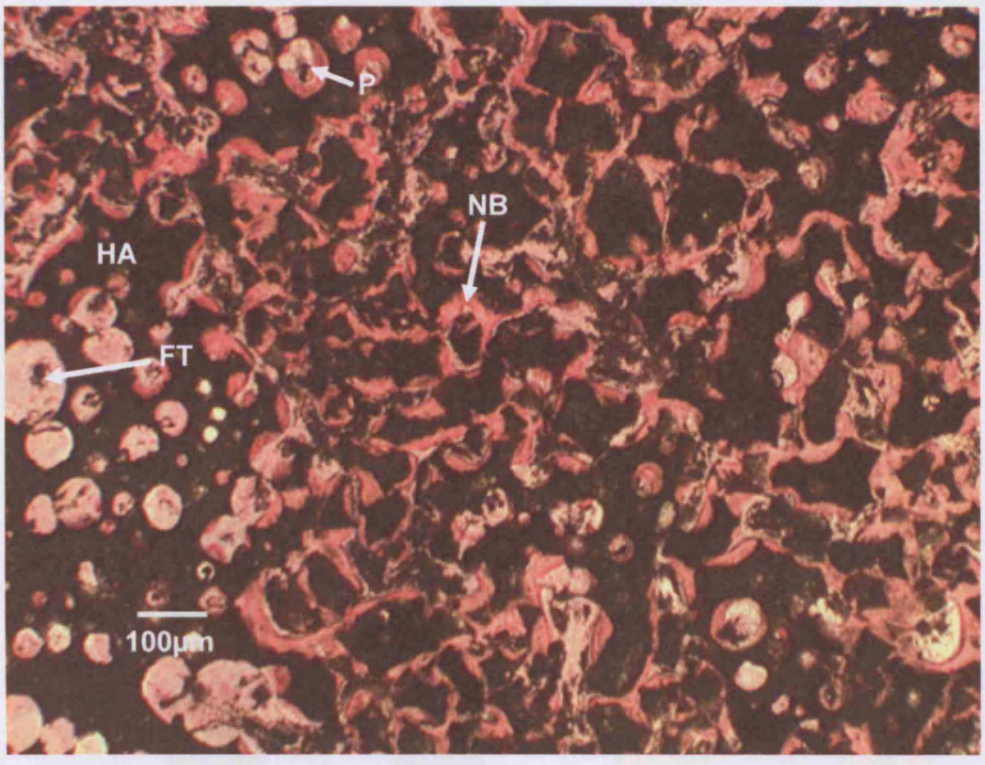


Figure 4.14 Image of HA taken at the middle region in the MSC group after 6 weeks *in vivo*. Remaining HA appears black with new bone (NB) within pores and following the contours of the HA particles. Fibrous tissue (FT) is also present.

4.3.3 Comparison of bone formation and graft remodelling in the allograft and HA groups.

4.3.3.1 Control Group

The quantity of new bone formation and remaining scaffold in the allograft and HA control groups was compared after six weeks *in vivo* and is represented in the graphs below (figure 4.15 and 4.16).

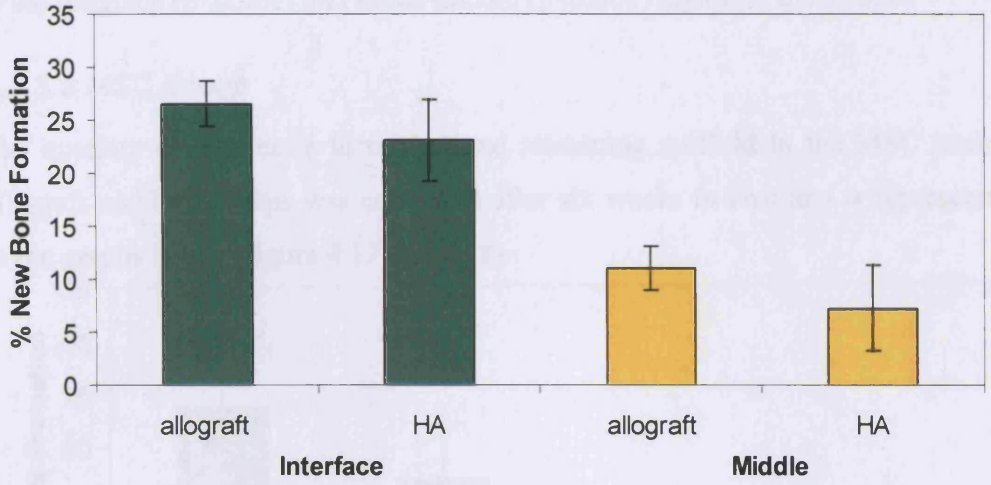


Figure 4.15 The graph represents percentage new bone at the interface and middle of the graft in ctrl allograft and HA scaffolds (±SE) after six weeks *in vivo*

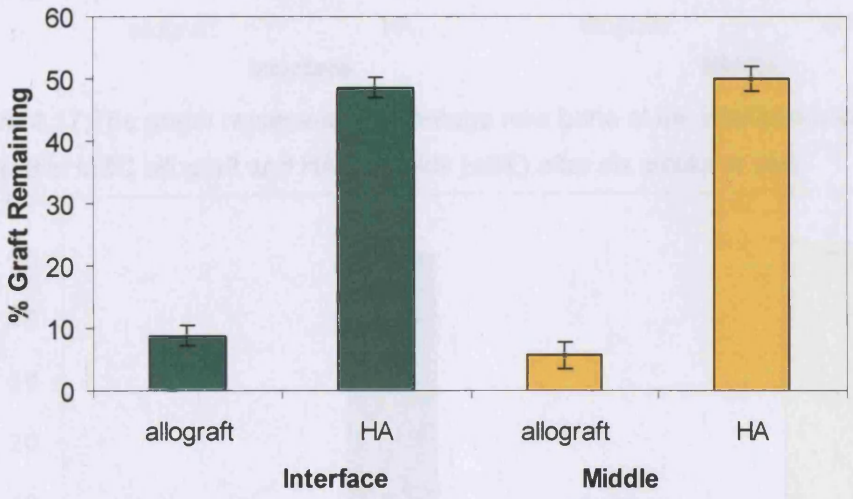


Figure 4.16 The graph represents percentage remaining graft at the interface and middle of the graft in ctrl allograft and HA scaffolds (±SE) after six weeks *in vivo*

Analysis of the control group reveals that at six weeks there was no statistical difference in the amount of new bone formation between the allograft and HA at the interface with 26.5% ± 2.1 SE and 23.1% ± 3.8 SE respectively (p=0.23) or the middle of the graft with 11.1% ± 2.1 SE and 7.2% ± 4.1 SE respectively (p=0.50) (figure 4.15). There is a significant difference between the amount of remaining allograft, at 5.7% ± 2.0 SE compared to remaining HA, at 50.1% ± 2.0 SE in the control group. There is significantly more remaining HA than remaining allograft at the interface (p<0.001) and in the middle (p<0.001) region of the sample.

4.3.3.2 MSC Group

The quantity of new bone formation and remaining scaffold in the MSC treated allograft and HA groups was compared after six weeks *in vivo* and is represented in the graphs below (figure 4.17 and 4.18).

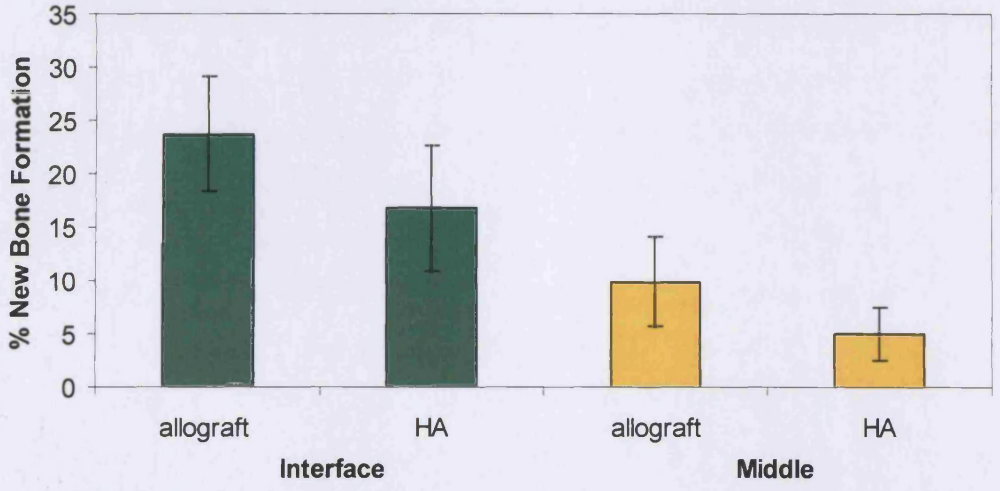


Figure 4.17 The graph represents percentage new bone at the interface and middle of the graft in MSC allograft and HA scaffolds (±SE) after six weeks *in vivo*.

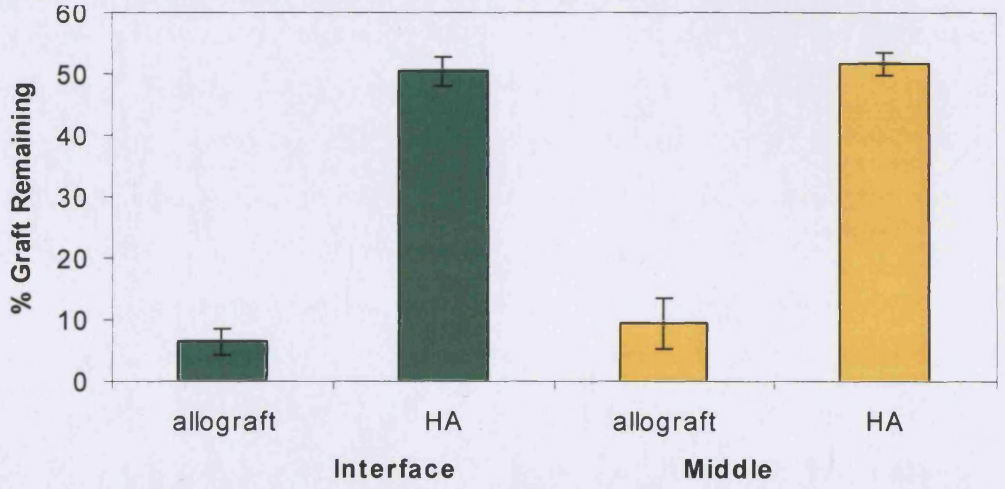


Figure 4.18 The graph represents percentage remaining graft at the interface and middle of the graft in MSC allograft and HA scaffolds (±SE) after six weeks *in vivo*

The MSC treated samples follow the same trend as the control group. Analysis of the MSC group reveals that at six weeks there was no statistical difference in the amount of new bone formation between the allograft and HA, with 23.6% ± 5.4 SE and 16.7% ± 5.9 respectively at the interface (p=0.35), or the middle of the graft with 9.8% ± 4.2 SE and 5.0% ± 2.6 SE respectively (p=0.50) (figure 4.17). There is a significant difference between the amount of remaining allograft compared to remaining HA in the MSC groups. There is significantly more remaining HA than remaining allograft, with 50.4% ± 2.1 SE and 6.4% ± 2.1 SE respectively at the interface (p=0.043) and 9.5% ± 4.1 SE respectively and 51.7% ± 1.9 SE respectively in the middle (p=0.043) region of the sample.

4.4 DISCUSSION

4.4.1 The effect of MSCs on new bone formation

The hypothesis that: **addition of MSCs to allograft or HA used in impaction grafting will increase new bone formation *in vivo*** was not proved. There was no significant increase in new bone formation or remodelling with the introduction of MSCs on impacted allograft or HA granules. In all groups more bone was formed at the graft-host interface than the middle of the graft, as would be expected from the invasion of osteoblasts and progenitor cells into the scaffold along with angiogenesis. The periphery of the graft is nearest to the source of nutrition and this is where the vessels would form first allowing osteoblastic activity. The middle of the graft becomes vascularised later and hence there is less time for bone formation during the experiment. This is described by early experiments where rabbit marrow cells were loaded into diffusion chambers, implanted into the peritoneal cavity that excluded host cells, but allowed nutrient diffusion in and out. Histological evaluation confirmed cartilage development in the middle of the chamber and bone apposing the internal interface which was encased by vasculature (Caplan 1991; Owen and Friedenstein 1988). Kruyt described new bone as forming from the outer HA surface toward the centre of the pores in ectopically implanted HA granules seeded with BMSCs in goats. A fluorochrome given at five weeks was seen directly on the HA surface, whilst the seven week marker was advancing toward the middle of the pore (Kruyt *et al.* 2003). This indicates that bone formation may occur along a nutrient diffusion gradient. However the granules were only $40mm^3$, larger constructs may not allow nutrient diffusion. Some studies suggest that once MSCs were seeded evenly throughout the graft, they would contribute to new bone formation throughout the graft, in the middle as well as the interface, even in larger constructs. Inner pore spaces of MSC-seeded HA constructs, used to assess tibial defect repair in sheep, were filled with new bone whereas new bone was limited to the outer surface of HA controls without cells on completion of the experiment (Kon *et al.* 2000). Similarly Bruder *et al's* study of the healing of critical size defects in the femora of dogs found that the MSC treated porous ceramic cylinders formed new bone throughout the implant, as well as at the cortex (Bruder *et al.* 1998b). In MSC seeded granules of

HA implanted in the paraspinalis muscles of goats Kruyt *et al.* found that ectopic bone was distributed unevenly throughout the granule, with a strong preference for the interior (Kruyt *et al.* 2003).

4.4.2 Comparison of bone remodelling in the allograft and HA groups

The hypothesis that: **Allograft will produce more bone than HA because of its osteoinductive nature** was unsupported by my work. Both scaffolds were shown to induce similar amounts of new bone formation. At six weeks there was no significant difference in the amount of new bone induced in allograft compared to HA in both the middle region of the graft and the interface region. There was a significantly greater degree of resorption in the allograft group, allowing more ingrowth of fibrous tissue. This makes the structural scaffold much more porous and therefore more likely to compromise the stability of the construct. The HA was not resorbed after 6 weeks and hence may be more stable.

As previously discussed in Chapter 1, autograft is the clinical 'gold standard' in bone grafting with the capacity to be osteogenic, osteoconductive and osteoinductive (Giannoudis *et al.* 2005). The most significant drawbacks of autograft include: donor site morbidity, extension of the surgical procedure, unnecessary invasive surgery and pain incurred at a healthy site, and limited quantity of graft from the donor site (Niklason 2000; Lane and Sandhu 1987). The most common alternatives to autograft are allografts. In the decade spanning from 1989 to 1999 the use of allograft has increased 15 times and accounts for one third of the bone grafts performed in the United States (Boyce *et al.* 1999). However, some osteoinductivity of allograft is limited when the graft is sterilised due to the necessity to remove cellular components that trigger the acute allograft rejection response (Giannoudis *et al.* 2005). Irradiation at 30 kGy has the least deleterious effects on the mechanical properties of bone with 25 kGy, the strength that was used in this Chapter and throughout my thesis, killing most bacteria (Pelker *et al.* 1983). Due to the increasing reliance on allograft instead of autograft, there is an increasingly limited supply available. Also there are complications associated with autograft as mentioned above and in Chapter 1 (Norman-Taylor *et al.* 1997).

HA has the advantage over allograft that it is unlimited in availability and there is no opportunity for disease transmission. The results in this chapter show that induction of new bone is comparable in both allograft and HA. In both cases new bone was formed within and in-between the graft particles, filling and bridging the gaps between them. This allows for the formation of an interconnected and cohesive scaffold. Substantial resorption of the allograft scaffold in this model initiated greater ingrowth of fibrous tissue. This effect seems to be associated with the rate of bone resorption and although the amount of new bone formed in the allograft filled defect was equal to that seen with the HA filled defect, fibrous tissue formation was increased in the former. The HA graft was not resorbed after 6 weeks. This makes the allograft/new bone composite less integrated than the HA/new bone composite and therefore more likely to compromise the stability of the construct. If the graft resorbs too soon after impaction grafting then there are implications for the stability of the hip replacement through subsidence (Gokhale *et al.* 2005; Eldridge *et al.* 1997). Based on the analysis in this Chapter it would be possible to use HA without allograft in an impaction model.

4.4.3 Sources of Error

There may be a variety of reasons why the MSC treated graft did not contribute significantly to new bone formation and remodelling. Since new bone was formed in MSC treated groups and there was no adverse effect as a result of the introduction of MSCs, they cannot be excluded as inducers of new bone. There are several instances where the methodology may not have been effective including problems associated with MSC differentiation, MSC seeding, graft morphology and loading, nutrient diffusion, surgery, length of study.

4.4.3.1 MSC Differentiation

MSCs in this experiment were maintained in culture as MSCs and were not differentiated into osteoblasts prior to implantation in the femoral defect. Ovine MSCs have been used in other studies and have contributed to new bone formation (Petite *et al.* 2000; Kon *et al.* 2000). In one study tibia diaphyseal resections were performed in four sheep. Two defects were filled with HA cylinders seeded with autologous bone marrow stromal cells (BMSCs) and two sheep received control samples without BMSCs. Morphological analysis showed a significant increase in

new bone formation of 50% over 7% in controls with no BMSCs (Chistolini *et al.* 1999). However, other large animal studies in goats have stimulated osteogenic differentation of BMSCs by introducing supplemented media prior to implantation of the construct. In Kruyt *et al's* initial ectopic study, described above, HA scaffolds were seeded and maintained in static culture with osteogenic supplemented media for six days prior to implantation. This resulted in significant bone formation, whereas devitalised, bone marrow and control samples did not produce any bone. This highlighted the importance of live cells in the contribution to new bone (Kruyt *et al.* 2003). A later study by Kruyt *et al.* in 2004 compared different combinations of culture conditions and grafts in order to optimise tissue engineering in goats. Treatment with osteogenic supplements did not influence bone formation (Kruyt *et al.* 2004b). However future studies in this thesis will include groups of MSCs treated with osteogenic supplements to promote osteogenic differentiation as well as maintained MSCs, as both culture techniques have been found to be successful in inducing new bone formation.

4.4.3.2 MSC numbers

In this Chapter MSCs were seeded onto graft at a concentration of 2 x 10^6 cells/cm^3. This concentration of cells was based on a published study where cell seeding density was investigated. After 3 and 6 weeks in culture, scaffolds seeded with 1 x 10^6 cells/cm^3 rat BMSCs showed similar tissue formation as those seeded with higher initial cell seeding densities. When initial cell seeding densities of 1 x 10^6 cells/cm^3 were used, osteocalcin immunolabelling indicative of osteoblast differentiation was seen throughout the scaffolds after only 2 weeks of *in vitro* culture (Holy *et al.* 2000). The use of cells at higher concentrations may be necessary in large animal models as metabolism is slower. The range of concentrations used in large animal models was: 8 x 10^6 cells/cm^3 (Kruyt *et al.* 2004a), 10 x 10^6 cells/cm^3 (Kruyt *et al.* 2003) in goats, 10 x 10^6 cells/cm^3 in a sheep model (Petite *et al.* 2000), and approximately 19 x 10^6/cm^3 were seeded in two canine models. However, it was suggested that some cells were not adherent and the numbers of non adherent cells were not detailed in either paper (Arinzeh *et al.* 2003; Bruder *et al.* 1998b). Due to time and limitations in space, the seeding density was restricted in the study in this Chapter. However, in future studies in this thesis higher seeding densities will be used.

4.4.3.3 Seeding Technique

The seeding technique used in this Chapter was optimised to deliver the MSCs to the defect site while the cells were in the most proliferative and durable phase of development after 4 days in culture as established in Chapter 2 (see Chapter 2, section 2.3.1.2). Choosing preoperative seeding of 4 days may have been a factor in limiting the contribution of new bone in the MSC seeded group. However, it was established in Chapter 2 that the cells remained viable and in a growth phase during static culture as shown by Alamar Blue™ analysis. Other authors have pre-cultured cells on graft (Kruyt *et al.* 2003). In addition, when constructs with cells incubated for 6 days were compared with samples cultured for 1 day after seeding, there was significantly more bone induced in the 6 day cultured constructs (Kruyt *et al.* 2004b). Others prefer intra-operative seeding of the graft by vacuum (Kadiyala and Jaiswal 1997), vacuum seeding with incubation at 37°C for 3 hours (Arinzeh *et al.* 2003) or by adsorbtion of cells onto the implant via a polymerising substrate such as fibrinogen (Kon *et al.* 2000) or fibronectin, where the HA and cell construct was implanted after 3 hours incubation (Bruder *et al.* 1998b). Any incubation period in these studies was merely to allow cells to adhere to the graft. The Kruyt group showed that precultured cells are not essential for bone formation, drawing a direct comparison between precultured and intra-operatively seeded cells. Amongst a range of seeding and culture techniques, cryopreserved goat BMSCs were either seeded onto constructs for 1 week or seeded intra-operatively in plasma and implanted ectopically. No significant difference in percentage bone area or bone contact was observed between these groups, but there was a significant increase over the control with no cells and bone marrow seeded calcium phosphate scaffolds (Kruyt *et al.* 2004a). Therefore an intra-operative seeding method is preferable to the complicated logistics of a preoperative method (Kruyt *et al.* 2004a) and will be used in future studies in this thesis.

4.4.3.4 Graft Morphology and loading

Most of the studies looking at TE constructs, seed MSCs onto blocks of porous ceramic bone substitutes. Some were much larger than the granules used in my study, being cylinders with a hollow central core (Arinzeh *et al.* 2003; Bruder *et al.* 1998b; Petite *et al.* 2000) or solid cylinders (Kon *et al.* 2000). One study utilised individual disks, relatively large in size compared to the granules used in this

Chapter (Bruder *et al.* 1998b; Kon *et al.* 2000; Kruyt *et al.* 2003; Kruyt *et al.* 2004c). There were also ceramic constructs of similar dimensions to those in this Chapter, but these only comprised of one or two granules (Kruyt *et al.* 2003; Kruyt *et al.* 2004b; Kruyt *et al.* 2004a). The large cylinders may have provided a stable scaffold for the cells compared to the granules used in my study. Kruyt *et al* (Kruyt *et al.* 2004c) showed less bone formation at the peripheries of ectopically placed TE constructs when compared to the middle. Micromovement on the outside of the implant was cited as a possible reason for the absence of bone there. Intrafragmentary strain within the impacted particulate graft may have played a role in the resorbtion of allograft and poor new bone formation in both TE constructs. The argument of increased interfragmentary strain in the location of the femoral condyle, where my implants were placed, is supported in the work of Duda *et al* (Duda *et al.* 1998). This study states that sheep bones are generally under compression with increasing ventral and lateral shear forces towards their ends. It is known that larger shear movement can lead to delayed union or non-union of fractures, so the femoral condyles may not have been an ideal location for a study analysing allograft granules. In addition, the femoral condyles are located adjacent to the joint surface where the bone end forms a saddle shape. The load is therefore more widely distributed over a larger cross sectional area containing solid trabecular bone than in the shaft of the femur, which is a cylinder of predominantly cortical bone surrounding marrow. Another consideration is that the femoral condyle design may not relate to the way a graft responds to force around a stiff metal implant. In a revision situation the graft surrounding the implant will be subject to stress shielding due to the stiffness of the implant.

4.4.3.5 Location and vascularisation of the graft

The diameter of the defects in my study were 15 mm. The cells in the middle of the graft would be 7.5 mm away from the nutrient supply in the adjacent host bone. Small animal models would not achieve this distance therefore the sheep model is more realistic to the clinical situation. One reason why new bone formation was not increased by MSCs could have been that the blood supply from the host in this situation is relatively accessible to the graft, whereas blood supply is drastically compromised in a revision situation. Therefore new bone formation by the MSCs may have been masked by osteoconduction from the host. However, an alternative

explanation could be that vascularisation is harder in larger grafts which are less favourable to fluid diffusion (Deleu and Trueta 1965). A low oxygen tension and poor nutrient supply may cause death of the newly transplanted MSCs.

Orthotopic locations for tissue engineering clinically sized constructs in large animals are hard to find in the literature and reflect that cell survival may be compromised by lower metabolic rates and slower vascularisation (Kruyt *et al.* 2003; Kruyt *et al.* 2004b). In a study on vascularisation of bone graft in the anterior chamber of the eye, penetration of vessels was faster in rats taking only 4 days, whilst in rabbits vessels penetrated only 3mm into the graft after 7 days (Deleu and Trueta 1965). However there are a few studies that demonstrate the successful application of tissue engineering of clinical sized constructs orthotopically in large animals (Petite *et al.* 2000; Bruder *et al.* 1998b; Arinzeh *et al.* 2003). Orthotopic and ectopic locations are directly compared in one study by Kruyt, although the implant dimensions are relatively small. BMSCs implanted on calcium phosphate ceramic scaffolds in the iliac crest of goats were compared with the same group implanted paraspinally. There was significantly more bone apposition on the available scaffold surface in the tissue engineered orthotopic site compared with control constructs at 9 weeks, but not at 12 weeks. However, there was a significant increase above the controls in the 12 week ectopic group (Kruyt *et al.* 2004c). One explanation they give is that the fracture healing response may have instigated phagocytosis. Another explanation they give is that the scaffold was very osteoinductive, overriding any effect of osteogenesis by donor BMSCs. This is less likely to be the case in my study as large amounts of fibrous tissue were observed in the allograft group. It is more likely that interfragmentary strain was responsible for fibrous tissue rather than new bone formation in my study.

4.4.3.6 Surgery

In my model the defects were only covered with the vastus medialis muscle following the implantation of the graft. This allowed for the ingrowth of fibrous tissue into the defects, which may have delayed or impeded new bone formation (Gosain *et al.* 2003b).

4.4.3.7 Length of the Study

In this study the samples were harvested at 6 weeks. Other studies looking at similar constructs harvested their samples at 8 to 9 weeks (Ohgushi *et al.* 1989; Kruyt *et al.* 2004c). The question remains whether more bone formation would have been seen at a longer time point. Other studies have shown more bone formation in the TE constructs at 4 weeks (Bruder *et al.* 1998b).

4.5 CONCLUSION

This study demonstrates that a bone substitute material does not need to be mixed with allograft and can be used on its own in an impaction grafting model. HA also has the advantage of being readily available and there are no risks associated with disease transmission. Additionally HA is more uniform in terms of quality than allograft bone and can be engineered to increase the strength of the graft particles for different applications. In a study by Van Gaalen, S. *et al*, porous ceramic implants seeded with or without autologous cells were placed either intramuscularly or paraspinally in Dutch milk goats. The implants with cells formed more bone than controls, however paraspinally there was no significant difference between groups and there was less new bone than at the intramuscular location. The reason for this was attributed to the slower diffusion of nutrients due to a less vascularised environment that may lead to cell death (Van Gaalen *et al.* 2004). In order to overcome the technical impediments described above and encourage new bone formation by tissue engineered constructs, the current technique was optimised in the next chapter by the use of scaffolds and cells in conjunction with plasma in a sheep model. An ectopic model was used to test the osteoinductivity of the cells, the cell seeding density was increased and the timescale was increased.

CHAPTER 5: New Bone Formation in an Ectopic Site

5.1 INTRODUCTION

In the study in Chapter 4 there was very little contribution to bone formation by autogenic MSCs on allograft impacted into condylar bone defects in sheep. The formation of new bone in the orthotopic site may have been induced by a combination of inductive factors in the graft, introduced autologous MSCs and osteoconduction from surrounding host bone. By carrying out the study in an ectopic site the true osseoinductive effect of the MSCs in regenerating bone can be elucidated without other complicating factors influencing bone formation, which are inherent in the bone marrow and bone matrix, such as osteoprogenitor cells from adjacent bone marrow and BMPs. This study therefore investigates the inductive properties of the cells by changing the implant site to the paraspinalis muscles, thus excluding the inductive influence of host bone. There could have been a variety of factors leading to a lack of increase in bone formation in the femoral condyl MSC group. The amount of bone could have been limited by other factors such as: 1) the use of MSCs where differentiated osteoblasts may confer an advantage in an impaction scenario, potentially reducing the time between lineage progression and therefore bone formation *in vivo*: 2) insufficient cell numbers in the *in vivo* model: 3) inappropriate preoperative seeding regimen: 4) high shear forces in the distal femur that possibly lead to non union of graft particles: 5) graft morphology: 6) large defect size leading to poor vascularisation of the graft:7) the unsealed defect allowing ingrowth of fibrous tissue and the limited length of the study at 6 weeks.

In this Chapter a model where an ectopic site is used which may indicate the osteoinductive nature of the cells and scaffolds. The cell seeding number and the length of implantation were increased in this study compared to the one reported in Chapter 4. The changes in methodology were primarily based on Kruyt's ectopic study in goats (Kruyt *et al.* 2004a). If new bone formation can be increased using tissue-engineered scaffolds in an ectopic site, it indicates the potential for regenerating bone in revision hip surgery.

Aim

The objective of this study is to investigate whether MSCs or osteoblasts contribute to bone formation in impacted allograft or an allograft and HA combination in an ectopic site.

Hypotheses

The sheep ectopic model allows eight sites to be used and therefore a number of comparisons between different scaffolds can be made. This model will therefore investigate the specific hypotheses that:

1. **Due to the osteoinductive effects of MSCs, impacted allograft seeded with MSCs will produce:**
    a. **More new bone than graft only.**
    b. **More new bone in contact with the graft than graft only.**
2. **Due to the fact that MSC derived osteoblasts are more committed to an osteogenic phenotype, impacted allograft seeded with these cells will produce:**
    a. **More new bone than allograft seeded with MSCs.**
    b. **More new bone in contact with the graft than graft with MSCs.**
3. **Due to the osteoinductive effects of MSCs, impacted HA/allograft (50:50) seeded with MSCs will produce:**
    a. **More new bone than impacted HA/allograft only.**
    b. **More new bone in contact with the graft than with HA/allograft only.**
4. **Due to the fact that MSC derived osteoblasts are more committed to the osteogenic lineage, impacted HA/allograft (50:50) seeded with these cells will produce:**
    a. **More new bone than impacted HA/allograft seeded with MSCs**
    b. **More new bone in contact with the graft than HA/allograft seeded with MSCs.**
5. **Due to the osteoinductive effect of MSCs, the unimpacted MSC seeded HA block will produce:**

a. **More new bone than the HA block only.**
b. **More new bone in contact with the graft than HA only.**

6. **Due to the osteoinductive nature of allograft, the impacted allograft will produce:**
    a. **More new bone formation than impacted HA/allograft (50:50) mix**
    b. **More new bone contact with the graft surface than impacted HA/allograft (50:50) mix.**
7. **Due to the osteoinductive nature of allograft, the impacted allograft seeded with MSCs will produce:**
    a. **More new bone formation than impacted HA/allograft (50:50) seeded with MSCs.**
    b. **More new bone contact with the scaffold surface than impacted HA/allograft (50:50) seeded with MSCs.**
8. **Due to the osteoinductive nature of allograft, the impacted allograft seeded with MSC derived osteoblasts will produce:**
    a. **More new bone formation than impacted HA/allograft (50:50) seeded with MSC derived osteoblasts.**
    b. **More new bone contact with the scaffold surface than impacted HA/allograft (50:50) seeded with MSC derived osteoblasts.**

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Graft

5.2.1.1 Allograft Preparation

Allograft was produced according to the protocol outlined in Chapter 2 (Chapter 2 section 2.2.2.).

5.2.1.2 HA.

The HA was provided in the form of ApaPore-60™ by ApaTech (ApaTech Ltd, Hertfordshire, UK) as described in Chapter 4, Section 4.2.2.2. HA granules 60/400 (2-5mm diameter) were used to investigate osteoinductivity under impaction. Blocks of the same material (5-10mm diameter) were used to investigate the effects of impaction. They will be referred to as HA blocks throughout the rest of my thesis.

5.2.1.3 Packaging of HA and allograft

Individual samples were measured by weight. The graft was size sorted using a sieve shaker. 2-5mm diameter allograft was measured into 3.5g aliquots. In the allograft group, 3.5g equated to 5mls. Eighteen 5ml aliquots of allograft were individually double packaged and heat-sealed. In the 50:50 allograft / HA group, 1.75g of allograft equated to 2.5ml. 1.75g HA equated to 3.5ml. The two scaffolds were mixed making a total of 6mls. Eighteen samples were double packaged and heat-sealed. HA blocks weighing 1g each and equating to 1ml were packaged as above. All samples were sterilised by gamma radiation at Isotron plc as in Chapter 2, Section 2.2.2.2.

5.2.2 Cell culture

5.2.2.1 MSCs

Bone marrow aspirates were obtained from anaesthetised sheep through the iliac crest. MSCs were isolated, expanded and used in the study, implementing techniques described in Chapter 2, section 2.2.1.3.

5.2.2.2 MSC differentiation assay for osteogenesis

In order to investigate the effect of incubating MSCs in osteogenic media on differentiation into osteoblast-like cells MSCs, were trypsinised from flasks and resuspended in 18ml of standard media at a concentration of 1 x 10^5 cells/ml. The experiment was performed in triplicate to test for repeatability. Six wells of three 24 well plates were filled with 1ml of this cell suspension. Each plate represented one timepoint and wells were analysed at 1, 7 and 14 days. Media was removed after 24 hours and 3 wells on each plate were given standard media, the other three wells were treated with osteogenic media (OM). The OM was made up of regular media of DMEM (DMEM, Sigma D-6429), with 10% FCS (First Link, West Midlands, UK), penicillin / streptomycin (1000iu/ml; 100µg/ml) (GIBCO Paisley, UK) supplemented with 10mM β-glycerophosphate (Sigma D1756), 50µg/ml Ascorbic Acid (Sigma A4544), and 1 x 10^{-8} M Dexamethasone (Sigma, D-2915). At the end of day 1 and at days 7 and 14, the following procedure was followed for the biochemical assay. The media in the wells was discarded and replaced with 1ml of distilled water. The plate was placed in the -70°C freezer to lyse the cells and then thawed in a 37°C incubator twice. The samples were thawed at 4°C overnight to allow regeneration of enzyme activity. Samples were assayed by an automated process in the COBAS BIO centrifugal analyser (Roche,UK). The reagent p-nitrophenol phosphate (RANDOX Laboratories Ltd, UK) was added to the samples, whereby ALP cleaved the phosphate group to give p-nitrophenol which is yellow at alkaline pH and is monitored in the COBAS BIO (Roche, UK) at 405nm. This gave a measurement of the activity of the enzyme.

The amount of DNA in each well was measured in order to normalise the amount of ALP produced in each well to the number of cells present. This is because the cell number varies due to differences in growth rate, therefore cell numbers would be different at each timepoint. The amount of DNA is proportional to the number of cells present. The fluorimetric dye Hoescht 33342 (Sigma, B2261, UK) is DNA-specific and binds to contiguous adenine-thymine base pairs, emitting fluorescence at a wavelength of 460nm. A standard curve was constructed using serial dilutions of calf thymus DNA (Sigma, D3664, UK). A 100µl sample of each lysate described above was added to 100µl of Hoescht dye and the serial dilutions for the standard curve and the cell lysates were measured using the Ascent plate reader (Labsystems, UK). The amount of DNA in the lysate was compared with the standard curve to give a value.

5.2.2.3 Analysis

The test was to determine if treatment of MSCs with osteogenic media had an effect on cell differentiation by measurement of the osteogenic marker ALP.

5.2.2.4 Preparation of osteoblasts for use in vivo

Osteoblasts were differentiated from MSCs. After passage 2, MSCs were cultured in osteogenic medium for 14 days. The medium was changed every 3 days. Before surgery, cells were trypsinised and resuspended at the time of operation in autologous plasma.

5.2.3 Suspension seeding

5.2.3.1 Peroperative seeding in plasma

MSC's and osteoblasts were trypsinised from the culture flasks and placed in 7mls of DMEM or osteogenic media respectively, for transport to the operating theatre. On arrival in theatre the cells were centrifuged at 2000rpm. The media was discarded and the cell pellet resuspended in plasma.

Blood at a volume of 45ml was drawn from the jugular vein of the sheep at the time of intubation and equally divided between three pre-chilled T75 flasks. The blood was pre-chilled to delay clotting time by placing the large surface area of the flasks on ice for 5 minutes. During this time the flasks were gently agitated by hand. Blood was then transferred to pre-chilled sterile universal tubes and centrifuged at 3700rpm for 5 minutes to produce a haematocrit (figure 5.1). Cells were resuspended in the plasma fraction at $10x10^6$ cells/ml of plasma. The plasma and cell suspension was seeded onto the scaffold and allowed to form a clot prior to implantation. Plasma alone was seeded on to scaffold in the control group.

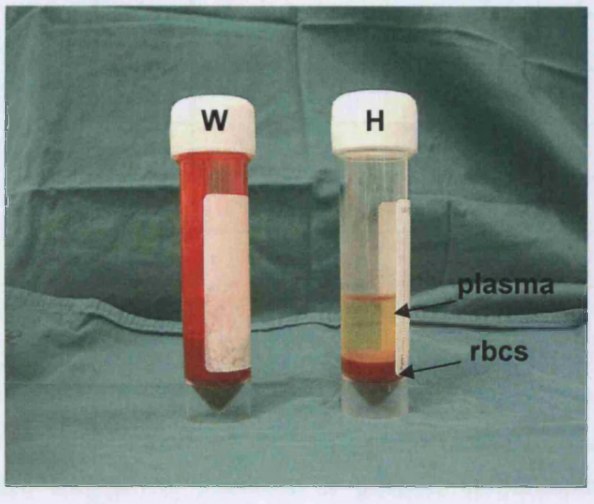
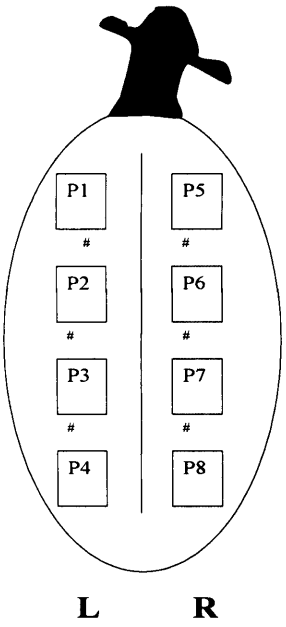


Figure 5.1: Universal containing whole blood (W) and universal containing a haematocrit (H) of plasma and red blood cells (rbcs).

5.2.4 Surgery

5.2.4.1 Preparation of the grafts

Six skeletally mature Mule sheep were used for the study. Eight groups were used to test three types of scaffold with six samples in each group. Each sheep received 8 scaffolds (8 scaffold groups) in the paraspinal muscles, which were: **1)** allograft control (allograft ctrl); **2)** allograft + MSCs (allograft MSCs); **3)** allograft + osteoblasts (allograft OB); **4)** 50:50 HA/allograft only (50:50 ctrl); **5)** 50:50 HA/allograft + MSCs (50:50 MSC); **6)** 50:50 HA/allograft + osteoblasts (50:50 OB); **7)** HA block (block ctrl); **8)** HA block + MSCs (block MSC). The MSC (MSC group) or osteoblast (OB group) were resuspended in autologous plasma at a concentration of 10 x 10^6 cells / ml plasma. Plasma was used at 0.9ml/g of scaffold to achieve full coverage of the graft with plasma. Three millilitres of plasma were added to each sample and allowed to form a clot. In the HA block groups, 1 ml of plasma was added to the control groups and 1 ml of autologous plasma with 9 x 10^6 MSC to the tissue engineered group. Sample position was varied between 6 sheep (figure 5.2).



	Scaffold Groups 1-6					
Pocket	**Sheep 1**	**Sheep 2**	**Sheep 3**	**Sheep 4**	**Sheep 5**	**Sheep 6**
P1	1	3	5	7	6	4
P2	2	4	6	8	7	5
P3	3	5	7	1	8	6
P4	4	6	8	2	1	7
P5	5	7	1	3	2	8
P6	6	8	2	4	3	1
P7	7	1	3	5	4	2
P8	8	2	4	6	5	3

Figure 5.2: Schematic diagram of positions of pockets in paraspinalis muscles of sheep. The table on the right describes assignment of scaffold groups within pockets:**1)** allograft control (allograft ctrl), **2)** allograft + MSCs (allograft MSCs); **3)** allograft + osteoblasts (allograft OB); **4)** 50:50 HA/allograft only (50:50 ctrl); **5)** 50:50 HA/allograft + MSCs (50:50 MSC); and **6)** 50:50 HA/allograft + osteoblasts (50:50 OB); **7)** HA block (block ctrl) and **8)** HA block + MSCs (block MSC).

5.2.4.2 Impaction

The required volume of autologous plasma with or without MSCs or osteoblast cells was added to each graft to form a clot. The clotted sample was transferred into a Delrin tube lined with a stainless steel mesh (figure 5.3).

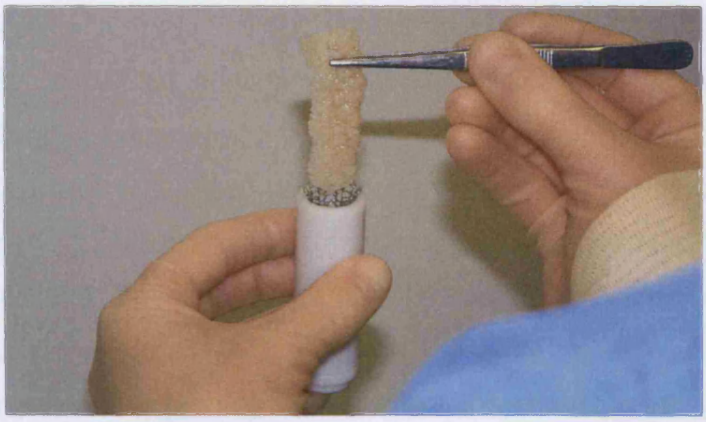


Figure 5.3 Plasma clotted around allograft being loaded into Delrin tube

The Delrin tube containing the sample was placed in the impactor and impacted twenty times with a force of 3KN (figure 5.4). The impacted sample was removed and the wire mesh was trimmed to size and sutured closed at both ends with an absorbable vicryl™ suture.

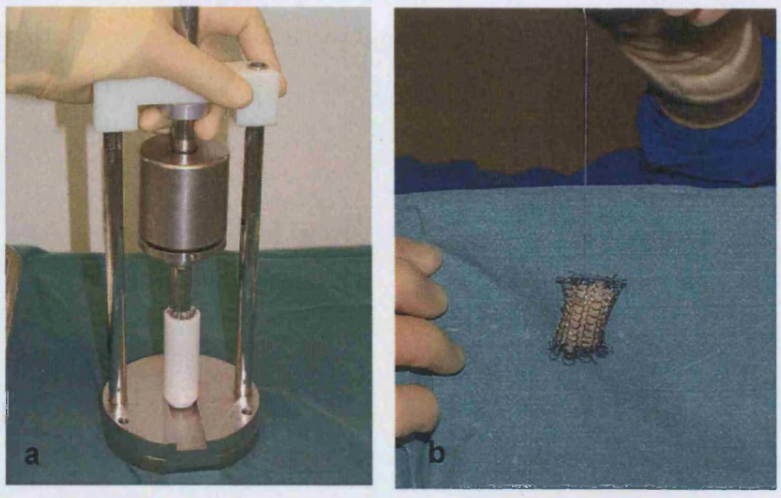


Figure 5.4a Impactor showing delrin tube and sample, **b** Allograft after impaction and suture

5.2.4.3 Operative procedure

All procedures were carried out in accordance to the Animals (Scientific Procedures) Act 1986. All procedures took place at the Royal Vetinary College, North Mymms as described in Chapter 4.

Six adult sheep were used. Each animal received 0.1mls/kg 2% xylazine intramuscularly 30 minutes prior to anaesthesia. The internal jugular vein was located with 2mg/kg ketamine and 2.5mg midazalam administered intravenously. An endotracheal tube was placed under direct vision and anaesthesia maintained using halothane 2%. One dose of intramuscularly amoxicillin (15mg/kg) was given. End tidal CO_2, Pulse oximetry and ECG monitoring took place through out the operation. A naso-gastric tube was placed to avoid aspiration. Each animal was placed prone on the operating table to allow access to both paraspinal muscle groups. Wool over both paraspinal muscles was shaved and skin prepared with betadine (iodine based) antiseptic solution. The area was draped with sterile sheets. Four skin incisions were made over the body of the paraspinalis muscles. Subcutaneous tissue was sharp dissected to the muscle belly. The epimysium was opened along the muscle fibres by sharp dissection followed by blunt dissection within the muscle belly to prepare a pouch (figure 5.5).

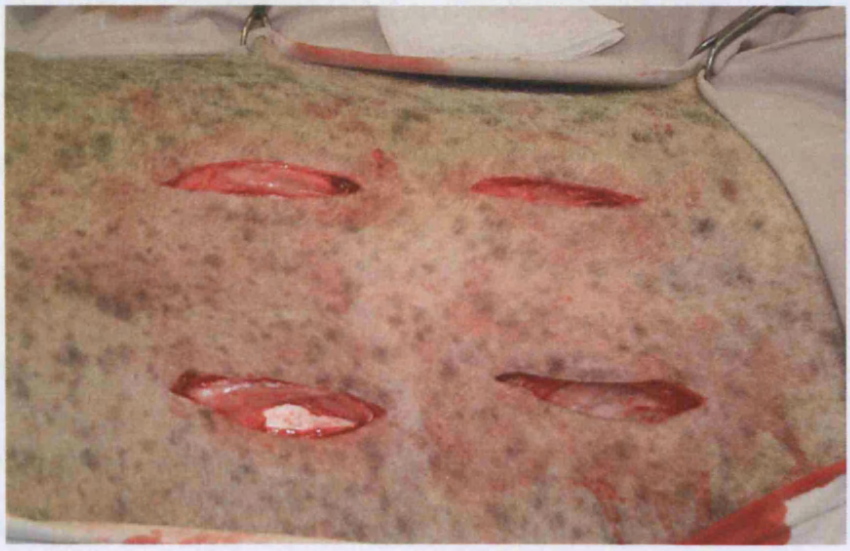


Figure 5.5 Four incisions made to access both paraspinalis muscle groups

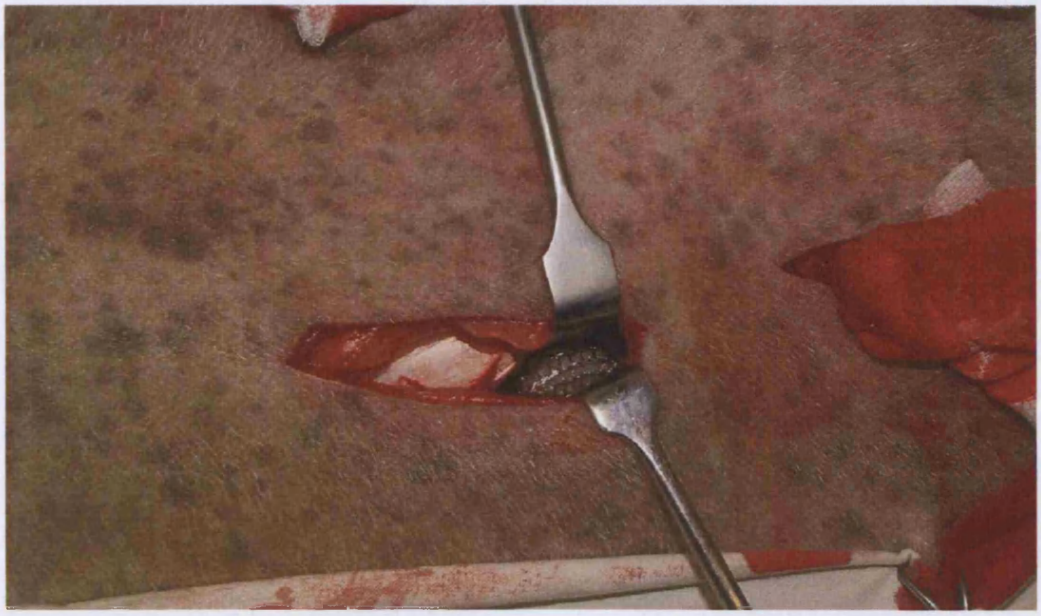


Figure 5.6 Image shows a metal cage containing graft that has been inserted inside the muscle belly. The muscle belly was opened along its fibres and a pouch made to insert the metal cage.

Each sample was placed and fully covered within the muscle belly (figure 4.5). The epimysium was closed with 2/0 non-resorbable ethylon suture and the wound closed in layers with 3/0 absorbable vicryl™ with interrupted vicryl™ to skin.

Pain relief involved buprenorphine administered intramuscularly (0.6mg per animal) intraoperatively and one-day post surgery. Animals were housed in individual pens for one week and then in a group pen thereafter.

5.2.5 Histology

5.2.5.1 Retrieval

All animals were sacrificed at twelve weeks. The animals were euthanized with an over dose of Phenobarbitone (50ml of 20% solution) intravenously. All eight samples were retrieved, with the attached muscle and placed in 10% buffered formal saline. The samples were then dehydrated with solutions of ascending concentrations of IMS (BDH laboratory supplies, UK) and then defatted with

chloroform to allow impregnation of LR white resin (Agar Scientific Ltd, UK) according to and slightly adapted from the protocol outlined in Chapter 4. Table 5.1 displays the protocol followed for each sample.

Process	Number of days
10% buffered formal saline	7
50% IMS 50% distilled water	1
70% IMS 30% distilled water	1
100% IMS (repeat step 3 times)	1
Chloroform (repeat Step twice)	1
100% IMS (change IMS twice per day)	3
50% LR white resin 50% IMS	2
100% LR white resin (under vacuum for 10 seconds) Apply vacuum every day	6
Cast in LR white resin (1 drop accelerator/10ml resin) Leave in fridge.	24 hours

Table 5.1 Protocol for processing of sample from retrieval to casting in LR hard resin.

5.2.5.2 Cutting thin sections

The samples were cast in resin in the same manner as those in Chapter 2, section 2.2.5.4. The samples were a similar shape and size to the *in vitro* samples in Chapter 2 therefore they were cut on the Exact diamond edged band saw (310 CP) in the same manner. However the samples were contained within the stainless steel mesh within the resin instead of Delrin tubes, so care had to be taken to grind the samples evenly as follows:

The samples were cut through the longitudinal axis of the sample to expose the area of interest using the DRAPER Band Saw. The sample was glued to a perspex slide using cryanoacrylate glue with the surface to be thin sectioned facing upwards. The next procedure was followed to level the sample. The exposed surface was marked in all four corners with permanent marker. The sample was ground on the grinding machine until the marks were removed and a flat surface was achieved using medium to coarse grit paper. Technovit glue was placed on the face of the specimen and a perspex slide was carefully pressed onto the glue until there were no air bubbles in the glue. The technovit samples were placed under UV light to cure the glue for 30 minutes.

A slice was cut on the EXAKT saw 1mm away from the technovit glued surface. This new slide was ground down to 100μm thickness using decreasingly coarse silicon carbide papers (from P240 to P2500). Sample thickness of the slide was measured using the micrometer. Sample thickness was evaluated using a light microscope. Once the correct thickness was achieved the sample was polished using P4000 paper on the grinder for 10 minutes, followed by 5 minutes polishing using aluminium paste.

All samples were stained with Toludine Blue for 15 minutes, which stains fibrous tissue blue, washed with water and dabbed dry with paper towel. Slides were then stained further using Paragon for 20 minutes, which stains new bone bright pink and older bone lighter pink, thus allowing differentiation between allograft bone and newly forming bone.

5.2.5.3 Histomorphometry

Slides were examined using a light microscope. Digital Photos were taken using a low power x4 objective through the central area of the sample. Image analysis was performed using Image Tool 3.0 (UTHSCSA Texas, USA). Total area of photos were analysed and area of new bone, remaining allograft, and remaining hydroxyapatite was quantified using polygons to select regions of interest. Percentage new bone formation was expressed as the amount of new bone over total area available for new bone generation. New bone in contact with the scaffold was determined as a percentage by defining whether bone or fibrous tissue

was in contact with the edge of the scaffold where it intersected with lines on an overlayed grid pattern of 500μm. Histological analysis was carried out using Image Tool 3.0.

5.2.6 Statistics

For the biochemical expression of ALP, an independent t-test was used to determine if there was a difference in the upregulation of ALP. Friedman two way ANOVA was carried out on data to examine any differences between scaffold groups. If a significant difference was found, a post hoc pair-wise Wilcoxon test was used to examine any differences between two groups. The Wilcoxon test alone was used to show differences between the groups. The statistical software SPSS (SPSS v10.1, SPSS Inc, Chicago, USA) was used for the analysis.

5.3 RESULTS

5.3.1 Results of MSC differentiation assay for osteogenesis

The results of the ALP assay at three time-points is plotted below (figure 5.7) (n=3 animals). At every time-point the enzyme activity is always greater in the osteogenically treated samples than the control MSCs treated with standard media. The greatest increase in enzyme activity is at day 14. The Independent t-test shows that ALP protein expression is significantly upregulated in sheep cells after 14 days in osteogenic supplements relative to the control (p=0.026). Therefore the osteoblasts used *in vivo* were defined as those treated with osteogenically supplemented media for 14 days.

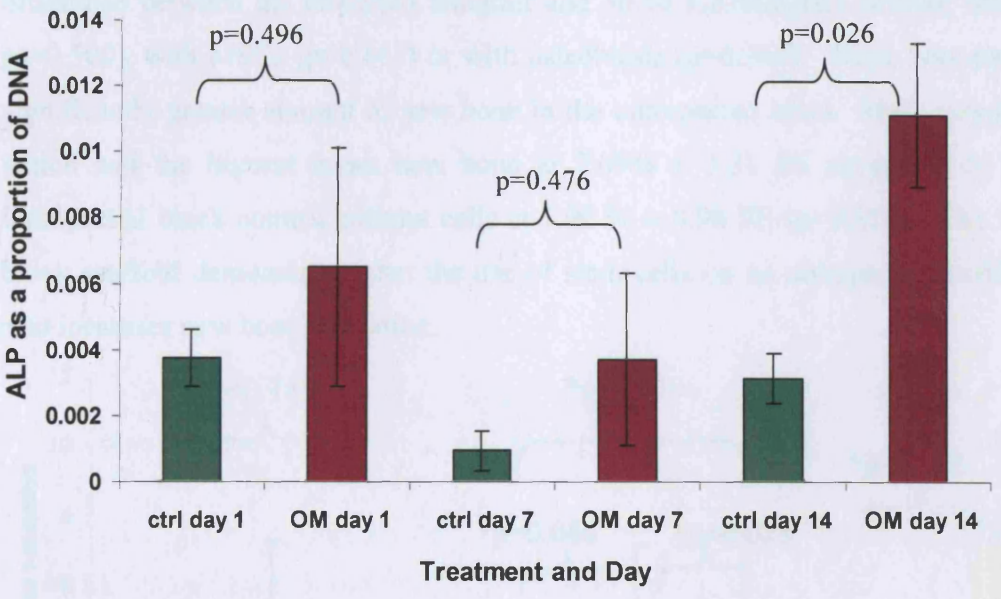


Figure 5.7 ALP as a proportion of DNA with p values for the Mann Whitmey U test ($p \leq 0.05$)

5.3.2 New Bone Formation

The graph below displays the mean new bone formation in all scaffold groups (figure 5.8). Scaffolds with MSCs incorporated into allograft, 50:50 HA/allograft and block groups displayed higher mean values of new bone area at 4.98% ± 2.28 SE, 5.15% ± 1.98 SE and 7.09% ± 3.31 SE respectively, compared with the controls at 2.24% ± 1.03 SE, 1.96% ± 0.79 SE and 1.96 ± 0.98 respectively. The osteoblast groups produced less new bone with 2.19% ±1.17SE in the allograft group and 0.82 ±0.26 SE in the 50:50 HA/allograft group.

Friedman analysis showed no significant difference between treatments in the allograft group, even though there was a greater amount of new bone in the MSC treated samples; however post hoc tests revealed significantly more new bone in the 50:50 HA/allograft MSC sample when compared to the 50:50 HA/allograft control (p=0.046) or 50:50 osteoblast samples (p=0.028). There was no significant difference between the impacted allograft and 50:50 HA/allograft without MSCs (p=0.500), with MSCs (p=0.463) or with osteoblasts (p=0.345). There was also a significantly greater amount of new bone in the unimpacted block MSC samples, which had the highest mean new bone at 7.09% ± 3.31 SE compared to the unimpacted block control without cells at 1.96 % ± 0.98 SE (p=0.028). The HA block scaffold demonstrates that the use of stem cells on an unimpacted scaffold also increases new bone formation.

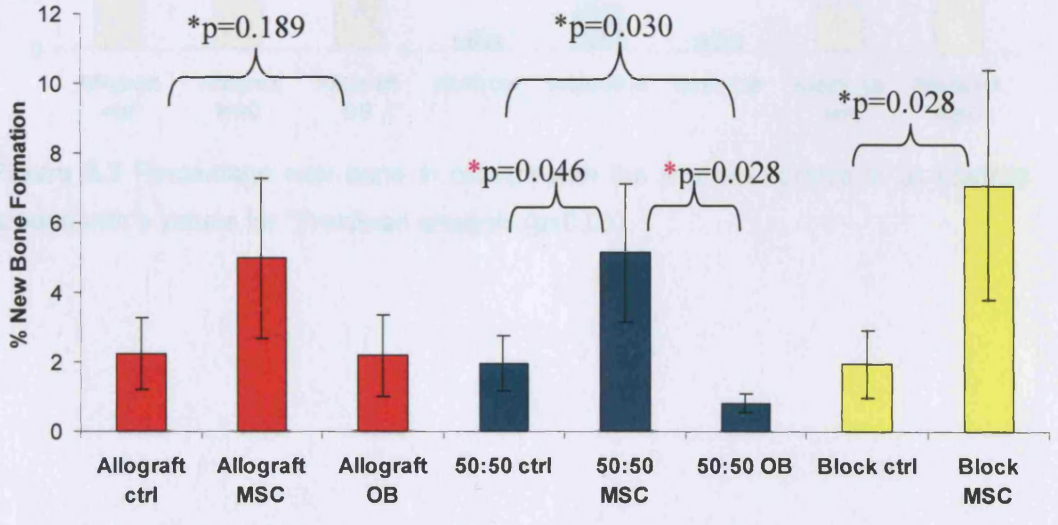


Figure 5.8 Illustrates New Bone Formation in all scaffold groups with p values for *Freidman and *Wilcoxon-signed rank (p≤0.05).

5.3.3 New Bone in Contact with Remaining Graft

MSCs produce more new bone in contact with the scaffold compared to the other groups (figure 5.9) with means of 39.52% ± 17.77 SE, 8.53% ± 3.16 SE and 14.15% ± 6.70 SE in allograft, 50:50 allograft/HA and HA Block groups respectively compared with the controls at 29.97% ± 14.80 SE, 2.66% ± 0.81 SE and 8.22% ± 2.43 SE respectively. The osteoblast groups produce the least amount of new bone in contact with the scaffold surface within each group with 21.64% ± 14.78 SE and 2.57 ± 1.14 SE in the allograft and 50:50 allograft/HA group respectively. However, there is no significant difference between treatments in any scaffold group in terms of new bone in contact with the scaffold surface. There was also no significant difference in bone contact between the impacted allograft and 50:50 HA/allograft without MSCs (p=0.116), with MSCs (p=0.225) or with osteoblasts (p=0.686).

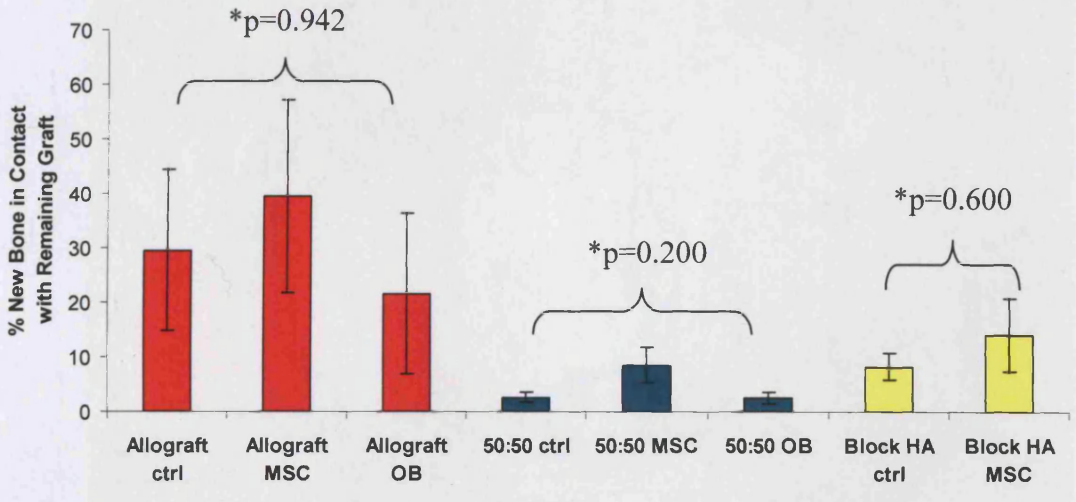


Figure 5.9 Percentage new bone in contact with the scaffold surface in all scaffold groups with p values for *Freidman analysis (p≤0.05).

5.3.4 Histology Images

5.3.4.1 Allograft images

The images reflect the data shown in the graphs. Bone forms directly without endochondral ossification (figure 5.10). New bone on the allograft forms in coastal areas around the graft pieces (figure 5.10a). New Bone was demonstrated throughout the slides and formed bridges between individual pieces of allograft (figure 5.10a-b). Osteocytes and lacunae were clearly visible. Surfaces of new bone were covered in a layer of osteoblasts, and surrounded by unmineralised osteoid. Osteoclasts demonstrated active areas of resorbtion. In one case there was evidence of new bone forming with no attachment to scaffold (figure 5.10c).

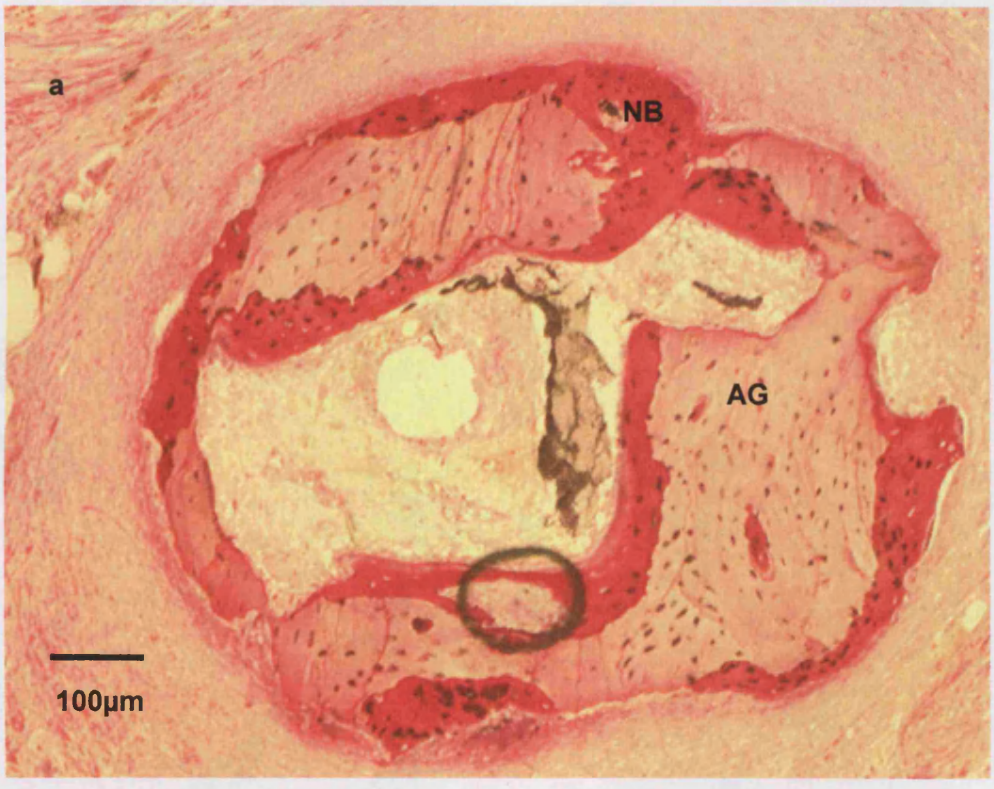


Figure 5.10a A typical example of allograft control

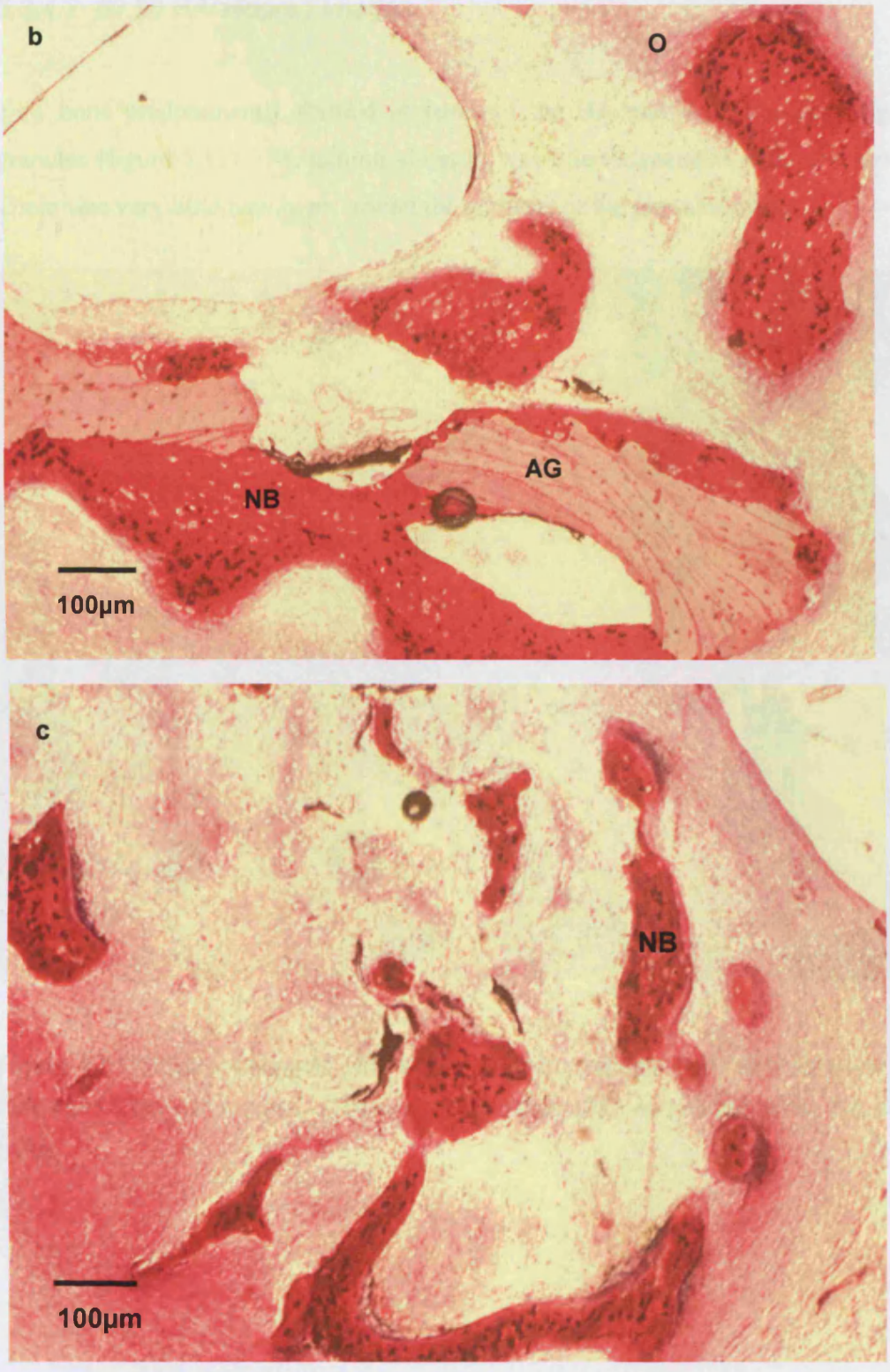


Figure 5.10b A typical example of allograft / MSC group; **c** showing areas of allograft (AG) bridged by new bone (NB) and centres of ossification (O) which appear to be unrelated to the allograft surface.

5.3.4.2 50:50 HA/allograft Images

New bone predominantly formed in pores of the HA and occasionally bridged granules (figure 5.11). Remaining allograft was often associated with new bone. There was very little new bone around the surfaces of the granules of HA.

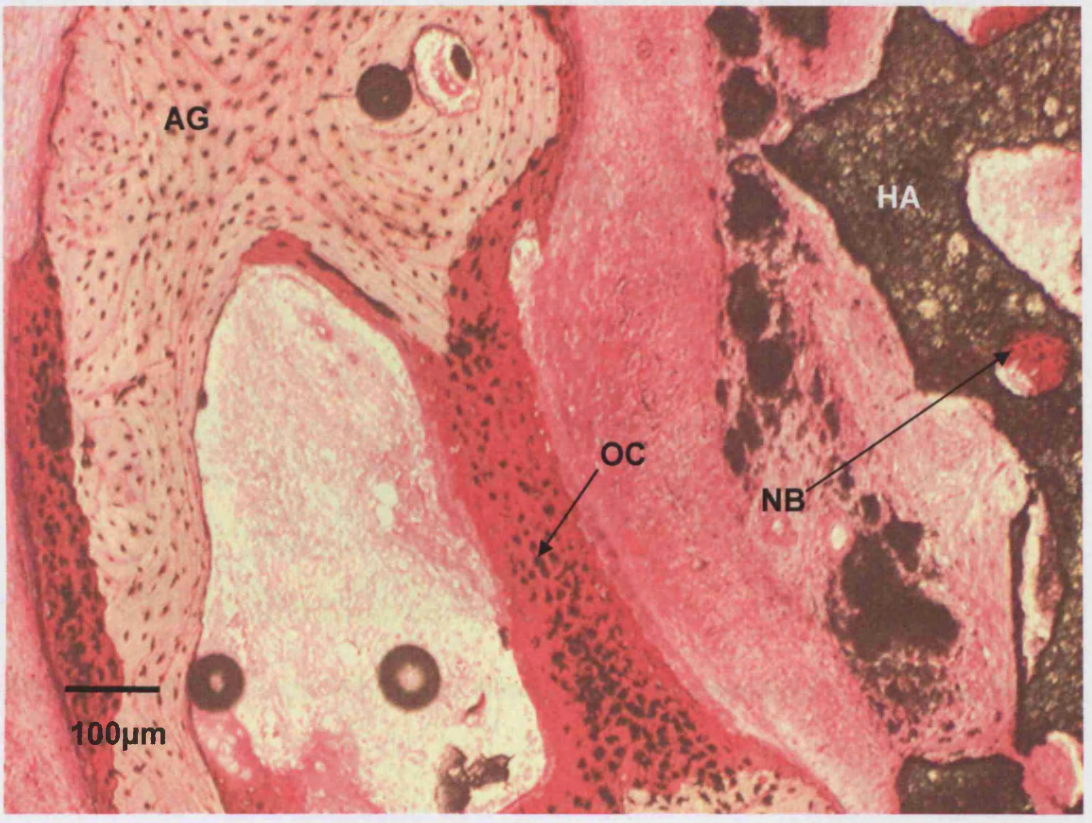


Figure 5.11 50:50 HA/allograft with MSCs. Hydroxyapatite (HA) with evidence of new bone (NB) within pores. There are osteocytes (OC) embedded within the new bone

5.3.4.3 HA Block

However in HA, new bone forms in isolated pockets within the HA pores (figure 5.12). Although the HA may exist in large areas, new bone in the HA does not tend to form around the surface of the HA granules as it does with allograft granules. It is not possible to know from these images whether the MSCs migrated within the pores of the graft or remained on the outer surface immediately after seeding.

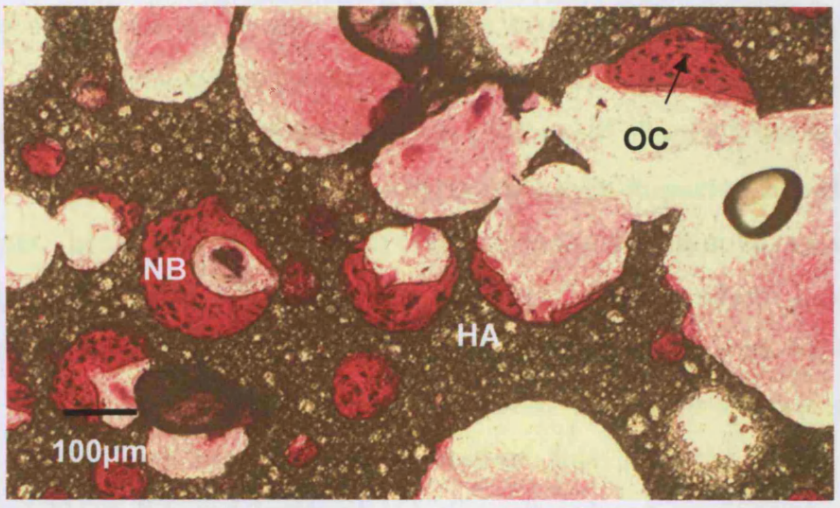


Figure 5.12 HA block with MSCs, and osteocytes (OCs) embedded in new bone (NB).

5.4 DISCUSSION

5.4.1 The effect of cells and graft on new bone formation

In the *in vivo* ectopic study, all groups showed some degree of bone formation. Control groups composed of allograft alone, HA/allograft and HA blocks showed little difference between each other. This indicates that HA is not only an osteoconductive material, it also shows some osteoinductive properties; while allograft does not demonstrate greater osteoinductivity than HA. The study rejects the hypothesis (6a) **Due to the osteoinductive nature of allograft, the impacted allograft will produce more new bone formation than impacted HA/allograft (50:50) mix.** In addition the hypothesis (7a) **Due to the osteoinductive nature of allograft, the impacted allograft seeded with MSCs will produce: more new bone formation than impacted HA/allograft (50:50) seeded with MSCs** was rejected. **In all cases of MSC groups compared with their control groups, there is an increase in new bone formation, but only the** MSCs in the unimpacted block HA and impacted HA/allograft induced significantly greater bone formation, **so the hypotheses (3a and 5a) were accepted.** However, the hypothesis (1a): **impacted allograft seeded with MSCs will produce more new bone than graft only** was not supported by this study. This may be because the allograft is osteoinductive so the osteoinductivity of the MSCs may have been masked by production of new bone derived from BMPs. The greatest amount of new bone formation on impacted graft was produced by the HA/allograft MSC group with 5.15% new bone. The study showed that t**he use of osteoblasts did not enhance the amount of new bone on any scaffold; therefore the hypostheses (2 and 4) were rejected.** Again there was no difference between different scaffolds seeded with osteoblasts. Finally, the hypothesis (8a): **Due to the osteoinductive nature of allograft, the impacted allograft seeded with MSC derived osteoblasts will produce more new bone formation than impacted HA/allograft (50:50) seeded with MSC derived osteoblasts** was rejected.

5.4.2 The effect of cells and graft on new bone contact

For bone contact MSCs do not make any significant difference for any of the scaffolds used, therefore the hypostheses 1b, 2b, 3b, 4b, 5b and also 6b, 7b, 8b are rejected. However, allograft scaffold either treated or not treated with MSCs results in more new bone contact when compared to corresponding scaffolds. The greatest amount of bone on impacted graft in contact with the scaffold surface was in the allograft MSC group at 39.52%. New bone contact with the scaffold was measured because bone formation has a close association with the scaffold surface. On histological examination of human cancellous grafting in a femur, new bone and osteoid made contact with dead allograft trabeculae (Ling *et al.* 1993). The mechanism of bone formation on HA was investigated by Chen, Q.Z. *et al* where strontium containing HA (Sr-HA) was placed in ilium defects in New Zealand white rabbits and examined using Transition Electron Microscopy (TEM) after 1, 3 and 6 months (Chen *et al.* 2004). New bone formed contact with the HA through the dissolution-precipitation mechanism. The mechanism is representative of calcium phosphates. First crystalline HA transforms into amorphous HA, producing an over saturated solution. This causes apatite crystals to precipitate in the presence of collagen fibres which become integrated with HA. Osteoblasts were also found near to the Sr-HA (Chen *et al.* 2004). Many studies report association of osteoblasts with HA. Collagen was reported to have condensed on HA, bone deposits in direct contact with HA and layers of osteoblasts line new bone matrix in Ripamonti's study in Baboons (Ripamonti 1991). Centripetal bone bonding, where bone is initiated at the HA surface and extends towards the centre of the HA pores is also reported in *in vivo* studies by Kruyt *et al.*, Okumura *et al.*, and Goshima *et al.* (Kruyt *et al.* 2003; Okumura *et al.* 1996; Goshima *et al.* 1991).

5.4.3 New bone area and new bone contact

My study shows that allograft/HA (50:50) and HA block produced more new bone area than allograft groups, some of the new bone forms in central islands without contact with the graft. However, there was more new bone contact on the allograft scaffold than either the HA/allograft or HA blocks. It is also evident from histology that new bone forms mainly in the internal pores of the HA. However, in

allograft, new bone forms predominantly on the external surface. The precipitation of amorphous HA is more likely in enclosed pores where it cannot diffuse away from the surface, encouraging centripetal bone formation, whereas in allograft new bone covers a larger surface area. This may be because the surface of allograft is more conducive to cell adhesion due to the presence of ECM, encouraging new bone to deposit on the surface.

This study has shown that MSCs influenced the production of new bone formation on impacted allograft and in an impacted HA/allograft. There was a significant increase in new bone in the impacted HA/allograft and unimpacted block HA group with MSCs. However there was no significant difference between the control and OB groups. There are several instances where the methodology may have influenced the results of the study, including implant location, scaffold geometry and location, length of the study, MSC numbers and seeding technique and osteoblast cells.

5.4.4 Implant Location

The study in this Chapter was adapted from the orthotopic methodology in Chapter 4 in order to investigate the hypothesis that sheep MSCs were osteoinductive. The location of the implants was moved to the paraspinalis muscles, thus removing the osteoinductive and osteoconductive influence of host bone. Other studies already described in this thesis have established that autologous sheep or goat cells seeded on bone graft substitutes can be osteoinductive in an ectopic site (Kruyt *et al.* 2003; Van Gaalen *et al.* 2004; Kruyt *et al.* 2004b; Kruyt *et al.* 2004a; Kruyt *et al.* 2004c). These investigations have extended to inductivity of xenogenic cells such as the transplantation of sheep cells into the subcutis of immunocompromised mice, where substantial new bone was formed in contrast with fibrous tissue in control samples (Kon *et al.* 2000). However, none have described osteoinductivity of the cells after impaction. The study in this chapter has established that HA and allograft alone and in combination are inductive, but also that the MSCs significantly increase the inductivity of the scaffold in the paraspinalis muscles, both in the impacted 50:50 HA/allograft group and in the unimpacted HA group. In my study, the amount of new bone ranged between 4.98% and 7.09% in the MSC group and 1.96% and 2.24% in the control group. The results from the

ectopic implants in Kruyt's studies give values of between 0.5 and 5.0% in MSC and osteogenically treated groups (Kruyt *et al.* 2004a), which are close to the quantities found in this chapter. Table 5.2 summarises the amount of new bone produced in ectopic studies in the literature and my study. The studies listed are those that most closely resemble the parameters used in this Chapter. The studies with the black asterix have the most in common with my study methods and also produce new bone in a similar range. From these studies and my study, the amount of bone produced in an ectopic site is low, but these models are still able to discern osseoinductive materials for other less effective scaffolds.

Location	Animal	Time (weeks)	Histomorphometry	Scaffold	Seeding technique	New Bone				Publication
						ctrl	MSC	OB	Bone marrow	
Ectopic Paraspinal	Goat	12	Percentage of available space occupied by bone	50-60% Biphasic calcium phosphate	Precultured	0.6 ± 0.8	N/A	10.2 ± 2.9	N/A	(Kruyt et al., 2004c)
*Ectopic Intramuscular Lumbar	Goat	12	Percentage of available space occupied by bone	Granules: *HA 60%	*Direct seed	N/A	0.5-1.0		N/A	(Kruyt et al., 2004b)
					Precultured	0	2.0-4.0		N/A	
				HA 70%	Direct seed	N/A	3.0-5.0		N/A	
					Precultured	0	9.0-13.0		N/A	
Ectopic Intramuscular Thoracolumbar	Goat	9	Percentage of available space occupied by bone	50% porosity HA/TCP cube	Peroperative	0.2	14.0	11.5	N/A	(Kruyt et al., 2004a)
					Precultured	0.7	11.2	11.7	4.2	
*Ectopic Intramuscular	Goat	12	Percentage of available space occupied by bone	Two granules: *HA 60%	Precultured	0	0	3.5 ± 4.0	0	(Kruyt et al., 2003)
				HA 70%		0	0	13.2 ± 15.9	0	
*Ectopic Paraspinalis muscles	Sheep	12	Percentage of available space occupied by bone	*Allograft	Peroperative	2.24 ± 1.03	4.98 ± 2.28	2.19 ± 1.17	N/A	Chapter 5
				*Allo/HA 60%		1.96 ± 0.79	5.15 ± 1.98	0.82 ± 0.26	N/A	
				HA 60%		1.96 ± 0.98	7.09 ± 3.31	N/A	N/A	

Table 5.2

*Impacted scaffold

*Studies that most closely resemble the parameters used in Chapter 5

5.4.5 Scaffold geometry and location

Most studies in the literature have used large, individually implanted ceramic blocks as scaffolds, rather than granules (Arinzeh *et al.* 2003; Bruder *et al.* 1998b; Petite *et al.* 2000; Kon *et al.* 2000). My study used allograft and HA granules ranging from 2-5mm in diameter in an ectopic location. A reason for changing the location of the graft to an ectopic site was that intrafragmentary strain is greatest in the femoral condyles (Duda *et al.* 1998). Work by Kenwright and Goodship provides evidence that micromovement that exceeds defined boundaries can be deterimental to the bone healing process. The study investigated the effect of load or displacement on fracture healing in the sheep tibia by keeping one parameter constant and manipulating the other by the use of an external fixator. Although small micromovement of 0.5mm assisted the healing process, displacement of 2mm was detrimental to mineralisation and fracture healing (Kenwright and Goodship 1989). This is supported by Lacroix *et al* who demonstrated that high shear strain loaded fractures, simulated in a finite element model, stimulated fibrous tissue, whereas lower strains were needed to produce cartilage and bone (Lacroix and Prendergrast 2002). There is evidence to suggest that the use of granules may be just as influential in disrupting optimal new bone formation as the implant location. The geometry of the granules may act independently from the effect of ectopic location, as Ripamonti found that bone induction occurred only in rods rather than granules (Ripamonti 1991). Geometry may have an influence in two ways. Firstly by accumulation of inductive factors within porous ceramics with cavities rather than solid ceramics (Yamaski and Sakai 1991). For instance, although my grafts are porous, inductive factors will be more likely to diffuse away from the small individual granules than from within large blocks, where inductive factors may be contained during resorbtion of the ceramic. Secondly, the granules may be more susceptible to interfragmentary movement because they are less stable than a block, causing disruption between the granules which may increase fibrous tissue formation (Ripamonti 1991). Therefore the use of granules as a scaffold in an ectopic site may also cause high levels of inter-granular strain and inhibit new bone formation. This may have prevented further quantities of new bone forming in the study in this chapter. Similarly, van Eeden found that bone differentiation was indicative of blocks of

HA rather than granules, suggesting that micromotion between individual granules, and granules and the adjacent bone interface may lead to fibrous union (van Eeden and Ripamonti 1993). However, in this study the use of granules was consistent as granules are more clinically relevant in impaction surgery than strut grafts or equivalent HA blocks. For this reason, a mixture of allograft, promoting osteoinduction, and hydroxyapatite, to provide a stable scaffold for osteoconduction, was used. Allograft alone was introduced for clinical relevance. In order to address the issue of interfragmentary strain, unimpacted HA blocks were used to compare my study with others, as well as to examine the osteoinductive effects of MSCs.

5.4.6 Length of Study

Sheep were sacrificed after 12 weeks to analyse new bone formation using tissue engineered bone graft in femoral condyle defects in chapter 4. Increasing the duration of the experiment was likely to produce more bone. The *in vivo* timeframe of 12 weeks was more representative of the length of studies in the literature compared to 6 weeks. For example experimental duration in large animals was 8.5 weeks (Kon *et al.* 2000), 9 weeks (Kruyt *et al.* 2004a; Kruyt *et al.* 2004c), and 12 weeks (Van Gaalen *et al.* 2004; Kruyt *et al.* 2003; Kruyt *et al.* 2004c). In these studies new bone was found in the tissue engineered constructs similar to those used in this chapter. The length of the study in this chapter of 12 weeks was sufficient as new bone was formed in tissue engineered samples and distinguishable from the amount formed in the controls.

5.4.7 MSC numbers and seeding technique

The number of MSCs used in this chapter was increased from the previous study in chapter 4. With reference to chapter 4, previous studies have used similar numbers of cells. The range of concentrations used in large animal goat and sheep models includes: 8 x 10^6 cells/cm^3 in an ectopic and orthotopic model respectively (Kruyt *et al.* 2004a; Kruyt *et al.* 2004c), 10 x 10^6 cells/cm^3 (Kruyt *et al.* 2003) in a goat ectopic model, 10 x 10^6 cells/cm^3 in a sheep orthotopic model (Petite *et al.* 2000). In this study, all groups were seeded with 9x10^6 MSCs/g of graft. In order to compare this to studies in the literature, the number of cells can be normalised to volume of graft, which was about 5cm^3 in allograft and allograft/HA granule

groups. Therefore the number of cells were 6 x 10^6 cells/cm^3. This concentration of cells was taken into account whilst considering time and space limitations in culture.

A peroperative seeding method was chosen for the study in this chapter, the reasons having been cited in the discussion section of chapter 4. To reiterate, in an ectopic study in seven adult goats, ceramic scaffolds were seeded with BMSCs either 7 days preoperatively or during surgery. Some pre-seeded samples were treated with supplemented media for ostogenic differentiation. Bone area (11.2 to 14%) and bone contact with the scaffold surface (22.8 to 27.3%), quantified by image analysis, was found to be similar in all tissue engineered groups regardless of seeding technique (Kruyt *et al.* 2004a). A possible reason why new bone may be increased by preculturing cells on graft is the production of extra cellular matrix (ECM). However, devitalised samples, with ECM only, contained significantly less bone than all the BMSC seeded groups, indicating that viable cells are more significant inducers of new bone than ECM (Kruyt *et al.* 2004a). It was therefore noted that for this study, preculturing was unnecessary and logistically more complicated than direct seeding at the time of surgery. A preoperative seeding method, which would involve cells cultured on a scaffold *in vitro*, would require labour intensive cell culture techniques. Maintenance of the cells would require more expensive cell culture technology, such as perfusion bioreactors, in order to sustain cell growth within the scaffold pores. It is also easier to standardise nutrient diffusion to the cells if they are transported to surgery in suspension and then seeded directly onto the scaffold at surgery.

5.4.8 Osteoblast cells

There was no increase in new bone formation or new bone in contact with the scaffold surface with the addition of osteoblasts on impacted allograft or impacted HA/allograft. In this chapter MSCs were differentiated into osteoblasts by supplementing the growth media with osteogenic supplements. The cells were defined by the presence ALP, a marker for osteogenic differentiation. Many other studies use a variety of markers, including ALP, to characterise osteoblasts or osteoblast-like cells, being an early marker for osteoblastic differentiation. Previous studies have shown that differentiation of sheep cells in the presence of

such supplements results in a significant increase in ALP activity determined by biochemical assay (Collignon *et al.* 1997; Torricelli *et al.* 2000). Osteoblastic differentiation was shown with increased alkaline phosphatase activity, production of osteocalcin and the formation of a mineralised matrix. In conflict with this data is the literature pertaining to sheep MSCs that have been shown not to express ALP in response to treatment with dexamethasone (Kon *et al.* 2000). In Kon *et al's* study this is not necessarily an adverse indicator of cell behaviour *in vivo* as HA ceramic cubes with MSCs improved on the amount of new bone in control mouse and sheep ectopic sites (Kon *et al.* 2000). However in the study by Kruyt, the effect of adding osteogenic media to MSCs cultured on scaffolds does not enhance new bone formation compared to untreated cells on scaffolds (Kruyt *et al.* 2004a). Ideally all the cells would be characterised using an array of markers to follow expression of osteoblastic proteins during differentiation. Another determinant of osteoblast expression is cbfa-1 (Runx2), a transcription factor that influences osteoblast differentiation (Ducy *et al.* 1997). Transcription of cbfa-1 could be measured using rt-PCR. However, the primers are not available for sheep sequences and the study is therefore beyond the remit of this thesis. In Chapter 3, section 3.2.2, cells were characterised using biochemical markers for ALP, osteocalcin and the histochemical stain for phosphate deposition, Von Kossa after 14 days treatment with osteogenic media. In this Chapter MSCs were treated with the same regimen as in Chapter 3, therefore characterisation was limited to ALP staining. The reason why osteoblastic cells derived from MSCs may not have contributed to new bone formation in this Chapter may be because MSCs cultured in osteogenic media differentiated into osteoblast cells that are known to have a higher metabolic activity and therefore require greater oxygen supply (Vacanti 2004). This could diminish their ability to survive in a low oxygen environment such as a large graft where nutrient diffusion would be slow in some areas, thus resulting in equivalent amounts of new bone as the controls seen in this chapter.

Further studies are underway in our Institution using MSCs in impaction grafting and a revision total hip replacement model. Further work is needed in characterising osteoblasts in sheep.

5.5 CONCLUSION

All groups showed some degree of bone formation. Control groups composed of allograft alone, HA/allograft and HA blocks showed little difference between each other. This indicates that allograft is not particularly osteoinductive. In all cases there is an increase in new bone formation when autologous MSCs are added. MSCs in the block HA, as well as in impacted allograft and HA/allograft, induced greater bone formation. The results suggest that if there is a clinical requirement for new bone contact with the scaffold surface, the scaffold choice should be allograft with MSCs, as this yielded the largest quantity at 39.5%. For example, implant stability may be more likely if bone is contiguous with the scaffold surface, thereby promoting osteoconduction and integrity with the implant surface. If the requirement is a large amount of new bone, a combination of HA and allograft with MSCs should be used. This study also showed no added benefit in seeding the graft with MSCs cultured in osteogenic supplements. The efficacy of using allograft with MSCs in a representative ovine clinical impaction model will be tested in the next chapter.

Summary:

- **MSCs increase new bone on any scaffold after impaction.**
- **There is no advantage of seeding osteoblasts on graft.**
- **Use a combination scaffold if areas of new bone are required and an allograft scaffold where contact of new bone with the scaffold is required.**

CHAPTER 6: New Bone Formation in a Revision Hip Replacement

6.1 INTRODUCTION

This chapter investigates whether bone formation can be enhanced using tissue engineered allograft in a model that approximates impaction allografting in a human THR situation. In chapter 5 MSCs were found to increase osteoinductivity in an ectopic site after impaction on an allograft as well as allograft / HA scaffold. Bone formation reported in Chapter 5 was relatively low. This may have been because the amount of bone formed in the ectopic site was directly related to the contribution of MSCs alone. In this chapter the number of cells was increased to further enhance new bone formation. The amount of bone produced in the intramedullary cavity is expected to be greater because of bone formation due to osteoconduction from the host bone and osteoinduction from native osteoprogenitors residing in the host stroma. In this study the contribution of new bone will not only be from osteoconduction, but also from the additional mechanical influence of the local environment and the pressure of impaction. Impaction grafting in a hemiarthroplasty sheep model represents impaction in a human situation, imitating the local mechanics occuring around a femoral stem and in THRs. In this way the ovine model reflects how the load is distributed from the implant onto the surrounding bone and allograft. The essential difference between this and the ectopic study in Chapter 5 is that impacted allograft surrounds an implant which is contained within the femoral cavity, resulting in residual stress. The load is therefore imparted to the prosthesis and results in increased bone stresses that may initiate a continuous repair process (Tägil 2000).

The main reason for the use of a sheep model in this study is that the proximal femur is similar to sclerotic bone, being very tubular and therefore akin to bone in a revision situation (Goel *et al.* 1982). The ovine hemiarthroplasty model was used by Blom *et al.* to investigate the compatibility of ceramic bone graft substitutes as allograft extenders for impaction grafting in the femur because of its similarity to the human clinical situation (Blom *et al.* 2005). Equally pertinent are hip joint forces that have been compared in sheep, dogs and man by telemetry using strain gauges attached to the femoral component in a THR. The magnitude and direction of hip joint forces in the sheep are comparable to those in humans and this is

another reason why sheep are a good model for femoral implants in the proximal femur (Bergmann *et al.* 1984).

Aim

The main objective of this part of my study was to regenerate new bone in defect areas adjacent to a hemiarthroplasty using impaction grafting with allogenic bone combined with either MSCs or osteoblasts derived from MSCs.

Hypotheses

1. **There will be a significant improvement in peak vertical Ground Reaction Force (pvGRF) in the MSC group compared to the control and osteoblast groups.**
2. **There will be a significant increase in new bone formation in the MSC group compared to the control and osteoblast group.**
3. **There will be a significant increase in new bone formation at the host allograft interface compared with the host implant interface within the control, MSC and osteoblast group.**
4. **There will be a significant increase in new bone formation in the MSC group compared to the control and osteoblast groups within the allograft implant interface or the host allograft interface.**
5. **There will be a significant increase in contact of new bone with the implant in the MSC group compared to the control and osteoblast group.**

The hypotheses postulate that MSCs will make more of a contribution to new bone formation, bone contact and function of the limb than osteoblasts or the control. This is because the results in the ectopic study in Chapter five showed that MSCs produced more new bone than the control, whereas osteoblasts did not. The same pattern occurred where contact of new bone with the graft was tested. Therefore it is expected that the trend will continue in the orthotopic model.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of allograft

6.2.1.1 Allograft Production

Allograft was produced according to the protocol outlined in Chapter 2 (Chapter 2 section 2.2.2.1).

6.2.1.2 Sieving the graft

The allograft used in the proximal region of the femoral component in this study was sieved to within the 3-5mm range. In this case the granule size for the proximal region was 2.4-4.76mm. This was the size of graft used in Chapters 2 and 3 for *in vitro* analysis. All the graft was placed at the top of the pile of sieves and shaken for half an hour until all the graft had fallen into its relevant sized sieve. A range of granules of larger size was used distally to the implant in order to fill the cavity. The graft required for the distal region included larger granules of >4.67mm mixed with 2.4-4.76mm granules, these grafts were bagged separately. Fine granules of 1-1.2mm were collected to fill the grooves of the implant so that contact of the graft with the implant was as close as possible to allow for bone ingrowth. This was double bagged separately. The allograft was gamma irradiated in a frozen state at 25KGrays (Isotron, Reading, UK). The allograft was stored at -20°C.

6.2.2 Cell culture

6.2.2.1 Obtaining bone marrow aspirates

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 at the Royal Veterinary College (RVC), North Mymms. Home Office licenses were held by all those taking part in the surgical procedure. Bone marrow aspirates were obtained from anaesthetised sheep through the iliac crest. MSCs were harvested and isolated in the study, implementing the standard tissue culture techniques described in Chapter 2.

6.2.2.2 MSCs

Cells in this study were used between passages 3 and 6 according to the techniques described in Chapter 2, section 2.2.1.3.

6.2.2.3 Differentiation of MSCs into osteoblasts

MSCs were differentiated using the same methods described in Chapter 3. The techniques used in Chapter 3 established that differentiation of the MSCs into osteoblasts had been achieved after 14 days in culture with supplemented media. Assays to determine a characteristic pattern of ALP regulation, a decrease in proliferation rate, increased osteocalcin and deposition of mineralised matrix converged on day 14 confirming an osteogenic phenotype. At P3 to P6 regular media was replaced with media supplements at concentrations: 10mM β-glycerophosphate (Sigma D1756), 50mM Ascorbic acid (Sigma A4544), Dexamethasone $1x10^{-8}$ M (D-2915 Sigma). Media was changed every three days for 14 days until cells changed to a cuboidal morphology. The cells were then harvested for transplantation in surgery.

6.2.3 Operative procedure

6.2.3.1 Intra-operative seeding of MSCs and MSC derived osteoblasts

Cells were seeded onto graft during surgery. A separate table was draped for all aseptic, peroperative seeding of graft. Either MSCs or osteoblasts derived from MSCs had been trypsinised from flasks, rinsed in regular DMEM and measured into aliquots of 100 x 10^6 cells in 10ml DMEM. Cells were transported in regular DMEM in universals to the RVC where the operation was to take place. Cells were seeded at a concentration of 10 x 10^6 cells per cm^3 volume of graft. Because each femoral cavity varied in size, extra aliquots of cells were brought and seeded at the above concentration in circumstances where more graft was required to fill the femoral cavity. The volume of allograft that was used for proximal impaction was measured out using a 10ml syringe.

6.2.3.2 Plasma preparation

Plasma was prepared in a similar manner to the protocol described in Chapter 5 section 4.2.3. Forty five millilitres of venous blood was collected from the jugular

vein of the anaesthetised animal, pre-cooled in T75 tissue culture flasks containing 15ml of blood per flask on a tray of ice, to prolong clotting time. Whole blood was then centrifuged at 3000 rpm for 5 minutes, creating a haematocrit with a plasma and erythrocyte fraction. Either MSCs or osteoblasts were centrifuged at 1500rpm and resuspended in 10ml of plasma aspirated from the hematocrit using a transfer pipette. The plasma with the cells was passed through the transfer pipette twice in order to achieve a cell suspension in the universal and then flushed through the pipette onto the 10ml of 2-5mm allograft for proximal impaction (medium graft/cell composite). As the plasma reached room temperature the rate of clotting around the graft increased, binding the cells and graft particles together (figure 6.1). This made the graft easy to handle during surgery. The same procedure was used to seed $2x10^6$ cells onto the 2ml volume of 1-1.2mm graft particles (small graft /cell composite). Plasma alone was added to allograft in the control group.

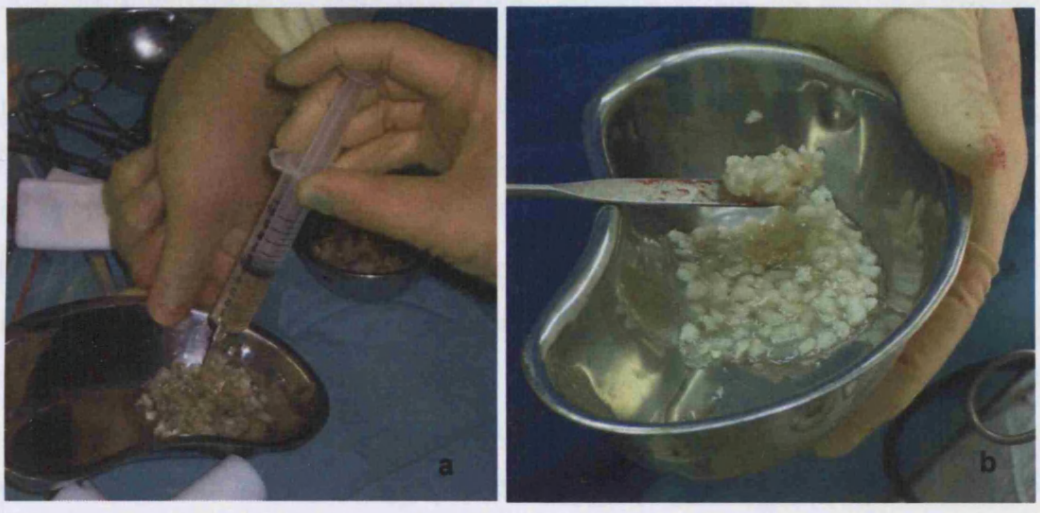


Figure 6.1a Illustrates the pre-cooled plasma in the liquid phase being dispensed onto allograft; b) illustrates the plasma clotted around the graft.

6.2.4 Surgery

All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 by those with valid Home Office project licenses. All procedures took place at the Royal Veterinary College, North Mymms as described in Chapter 3.

There were 15 adult sheep in the study divided into **3 groups** with **five sheep** in each group. The groups were:

1) **Allograft control without cells**
2) **Allograft seeded with MSCs**
3) **Allograft seeded with osteoblasts differentiated from MSC origin**

All animals underwent an uncemented hemiarthroplasty through an anterior-lateral approach using HA coated hip stems specially designed for the sheep. Once anaesthetized, an area over the lateral aspect of the right leg extending proximally over the pelvis to the proximal knee, was shaved. Betadine (broad spectrum topical iodophor microbicide) surgical scrub and alcoholic solutions were used to clean and decontaminate the skin. On the operating table the animal was positioned presenting the right lateral side. An area around the intended incision was draped. A skin incision about 10cm adjacent to the greater trochanter was made, where the tip of the greater trochanter was at the mid region of the incision. The incision was 2cm anterior of the greater trochanter. The septa between the fascia lata and the glucobiceps muscle was divided, the mid gluteal muscle was retracted and the deep gluteal muscle was sectioned as close as possible to its insertion point on the neck of the femur. The synovium was divided using a T shaped incision. The head of the femur was located and the ligamentum teres was divided whilst retracting the femur (figure 6.2). If possible, at this stage the hip was dislocated but more often the femoral head was resected and removed from the joint after resection.

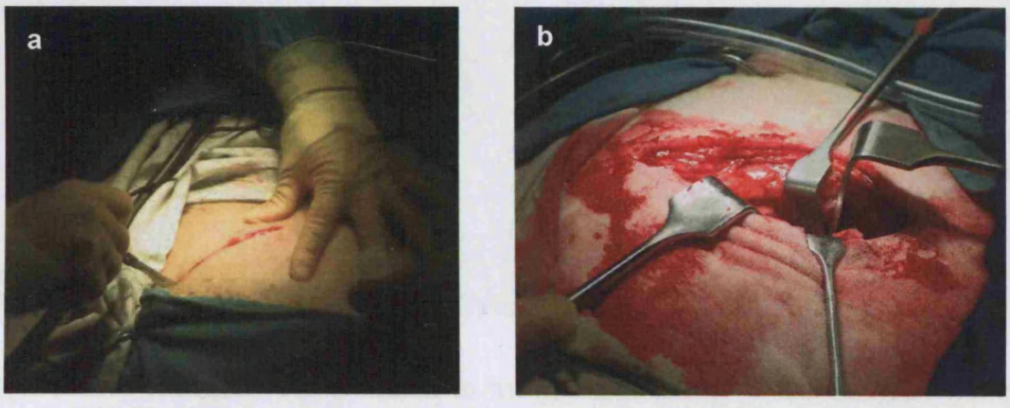


Figure 6.2a The incision placed adjacent to the greater trochanter; **b** The ligamentum teres was divided whilst retracting the femur.

The marrow cavity of the femoral canal was completely debrided of all fat and trabecular bone by curretting and flushing the cavity with saline, using suction to remove debris until the appropriate sized reamer could be passed down the intramedullary cavity. This left a radial space of at least 2-4mm around the stem to fill with allograft (figure 6.3).

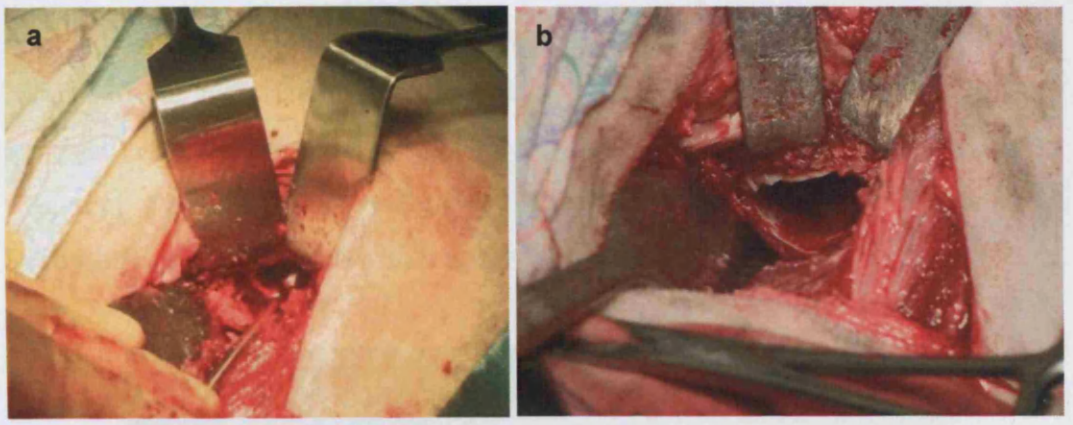


Figure 6.3a Trabecular bone of the femoral canal being curretted; **b** The final size of the radial space left after curetting.

On a guide rod a threaded intramedullary cement restrictor was inserted 3cm below the final position of the stem. The distal impactor was threaded over the guide rod and allograft was impacted onto the plug using a hammer on the distal impactor. Medium and large sized graft was impacted into the distal region of the femoral canal (figure 6.4). The volume of graft used in the distal region ranged from 15-25ml as measured in a syringe.

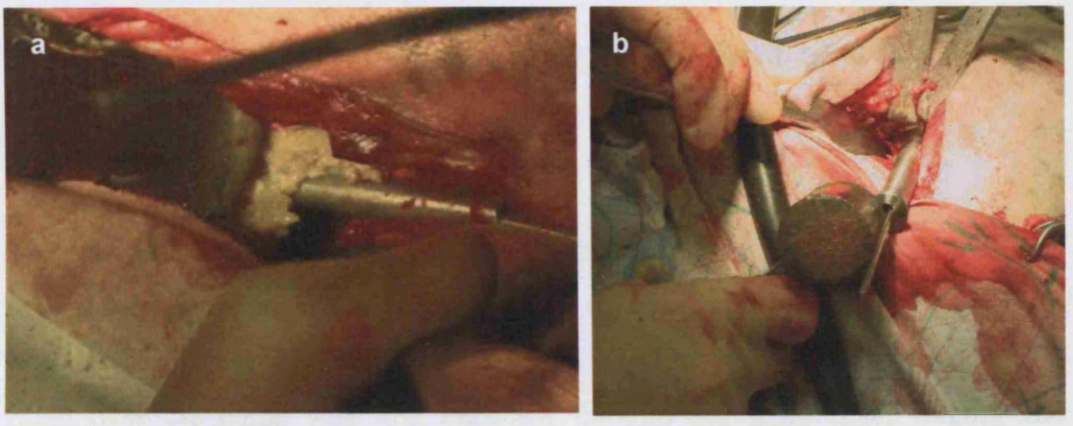


Figure 6.4 Allograft being added to the femoral cavity; **b** the allograft being impacted with a hammer on the distal impactor

6.2.4.1 Femoral and head components

The femoral stem was made by Stanmore Implants Worldwide Ltd, Stanmore, Middx, UK, to fit sheep femoral geometry. This allowed for a gap surrounding the implant for impaction allografting. The centre of the femoral head was matched to the anatomical centre of the sheep femur. The stem was titanium alloy and was fitted with a 25mm diameter modular cobalt chrome (CoCr) head. The neck length could be selected by choosing an appropriate femoral head supplied by Biomet Merck Ltd. (Biomet Merck Ltd., Swindon, Wiltshire, UK). The collared proximal stem was grooved to a depth of 2mm and a width of 2.5mm. The stem was sent away for HA coating at Plasma Biotal Ltd (Plasma Biotal Ltd, Derbyshire, UK). The stem was autoclaved prior to surgery.

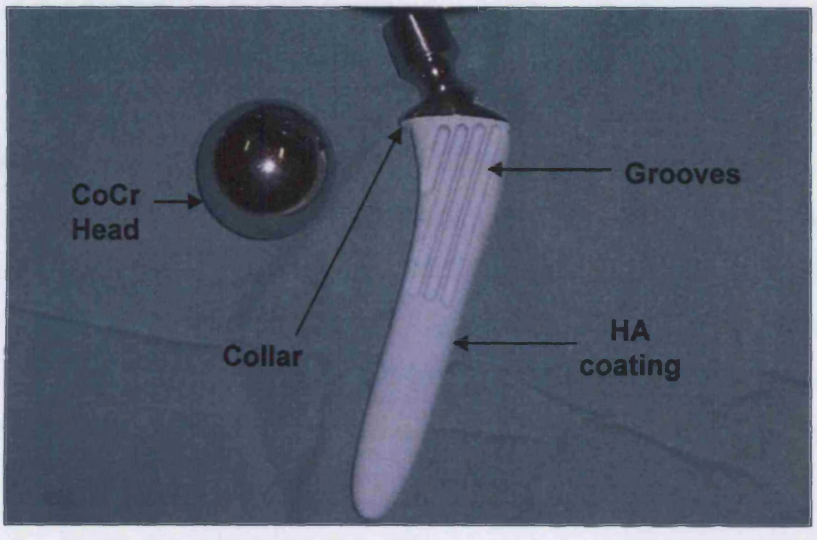


Figure 6.5 Titanium alloy femoral stem with proximally located grooves and a CoCr modular head. The stem is coated with HA.

6.2.4.2 Addition of graft/cell composite

The medium graft/cell composite was added proximally around a tube which fitted over the guide wire. After loose packing of the graft, the tube was removed leaving a small hole in the centre of the graft. A small sized femoral impactor was fitted over the guide wire and impacted through the small hole, forcing and impacting the surrounding graft onto the endosteal surface.

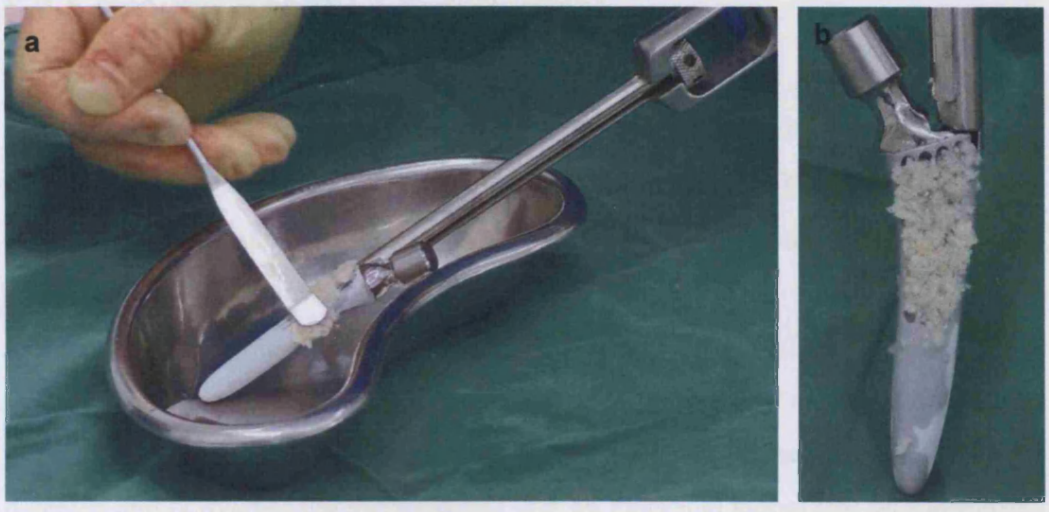


Figure 6.6a The small graft/cell composite set with plasma being pressed into the grooves of the femoral implant; **b** the femoral implant with the graft/cell composite completely filling the grooves of the implant.

If necessary, at this stage more graft was introduced around this small phantom which was then removed. A larger phantom was impacted before impacting the femoral stem. The small graft/cell composite was pressed into the grooves of the HA coated femoral stem before insertion (figure 6.6). The modular femoral head, 25mm in diameter with appropriate length, was placed onto the trunnion and secured by a gentle tap. The hip was reduced; the wound was washed and closed.

6.2.4.3 Closing the wound

The wound was closed in layers with absorbable vicryl™ sutures. The muscle apposed with 2 or 3 interrupted sutures, continuous closure of the deep fascia and dermis, with interrupted sutures to close the skin. Post-operatively the animals were recovered in sternal recumbancy and housed in individual pens for 24 hours.

6.2.5 Ground Reaction Force (GRF)

Sheep walking was carried out in accordance with the Animals Scientific Procedures Act 1986 by those with valid Home Office project licences. Ground reaction forces (GRFs) were measured in the sheep using a Kistler™ force plate 9287 (Kistler Instruments Ltd., Hampshire, UK). The ground reaction force is a measurement of extent of recovery of the operated limb and is therefore a reflection of the functionality of the hip joint after insertion of the implant. Sheep were guided through a gated walkway to achieve heel strike on the centre of the force plate. The piezoelectric transducers mounted underneath the force plate detected and converted the force exerted into an electrical signal that was amplified by the Kistler Charge Amplifiers type 5001 (Kistler™, Biomec Ltd, Hampshire, UK) and recorded. The peak vertical GRF (pvGRF) from 12 heel strikes of the right hindlimbs was collected pre-operatively and 12 weeks post-operatively. After pvGRF was collected the sheep was weighed on the force plate in Newtons and the pvGRF was normalised to body weight. The mean post-operative GRF of the right limb was then expressed as a percentage of the mean pre-operative pvGRF of the right limb.

6.2.6 Histology

After twelve weeks the sheep were euthanised with Phenobarbitone (50ml of 20% solution) intravenously. Femurs were removed and fixed in 10% buffered formal saline. Radiographs were then taken of the removed femora. Incisions, using a band saw, were made at 2cm intervals on alternate sides of the femur down to the level of the plug to ensure complete penetration of fixing and embedding reagents. The samples were then dehydrated with solutions of ascending concentrations of industrial methylated spirits (IMS) (BDH laboratory supplies, UK) and then defatted with chloroform prior to embedding in LR white resin (Agar Scientific Ltd, UK). The following protocol was observed:

Process	Number of days
10% buffered formal saline	7
50% IMS 50% distilled water	3
70% IMS 30% distilled water	3
100% IMS (repeat step 3 times)	3
Chloroform (repeat Step twice)	1
100% IMS (change IMS twice per day)	3
50% LR white resin 50% IMS	7
100% LR white resin (under vacuum for 10 seconds)	14
Cast in LR white resin (1 drop accelerator/10ml resin). Leave in fridge	24 hours

Table 6.1 Histology protocol for femurs

6.2.6.2 Cutting sections

After casting the specimens in resin, sections were cut through the transverse plane in three regions of the bone: 'proximal', 'mid' and 'distal' from the medial neck line. Radiographs were used to determine the position of each cut. The proximal section was cut 5mm from the medial neckline, the distal section was cut 5mm from the tip of the implant and the mid section was established halfway between these two points (figure 6.7). The 200μm sections were made on perspex slides after sticking samples down with Technovit glue. The sections were then ground to a thickness of 100 μm using the Exakt micro-grinding system and polished on the Motopol 2000 (Bueler, Coventry, UK). Prior to histomorphometric analysis of the sections they were stained with Toludine Blue for 10 minutes (which stains fibrous tissue blue) and Paragon for twenty minutes (which stains new bone bright pink and older bone a lighter pink).

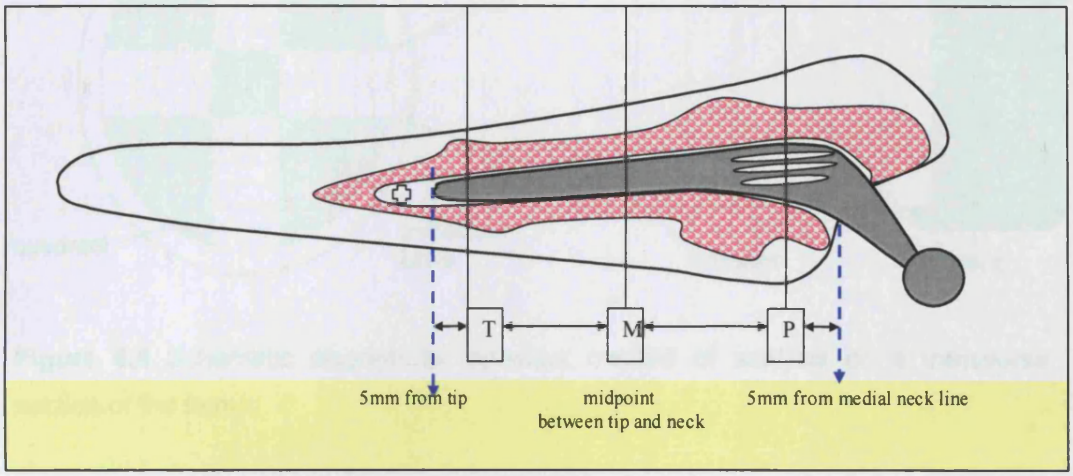


Figure 6.7 Cutting regimen for harvested femurs. T=Tip section, M=mid section, P=Proximal section. Vertical lines represent cutting planes.

6.2.7 Histomorphometry

6.2.7.1 New and remaining bone

New bone and remaining allograft were quantified by area using the KS300 computer program. Images were taken in anterior-posterior and medial-lateral planes from 4 quadrants in distal, medial and proximal sections. All images were taken using a x4 objective lens. New bone was scored in transects within each quadrant that extended from the inner surface, defined as the closest field to the implant, to the outer surface, defined as the closest field to the cortex (see figure 6.8). Percentage new bone formation was expressed as the amount of new bone over total area available for new bone generation.

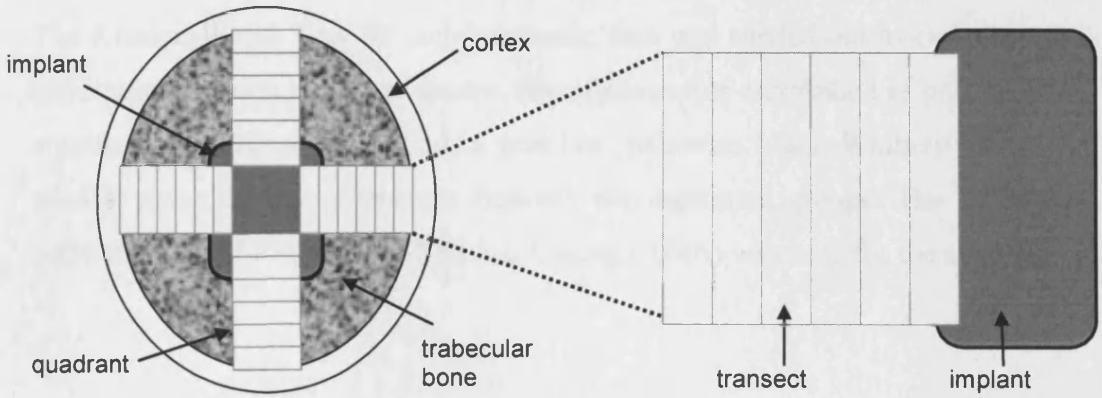


Figure 6.8 Schematic diagram to represent method of analysis on a transverse section of the femur.

6.2.7.2 Contact of new bone with implant interface

Contact of viable bone with the implant interface was quantified by counting the number of times lines on a 500 micron grid intersected with new bone and the implant. A grid of parallel lines was superimposed on the computer screen image of the implant-bone interface. Bone contact along the entire contour of the implant surface was determined.

6.2.8 Statistics

6.2.8.1 GRF

The peak vertical component of the GRF (pvGRF), measured in Newtons, was normalised by body weight (Fmax/weight) and the right post-operative GRF for the hindlimb was expressed as a percentage of the right preoperative hindlimb. The Kruskal-Wallis Test for non-parametric data was carried out to examine any differences between treatment groups. Significance was established as $p \leq 0.05$. If a significant difference was found, a post-hoc, pair-wise Mann-Whitney U Test was used to examine any differences between two treatment groups. The statistical software SPSS (SPSS v10.1, SPSS Inc, Chicago, USA) was used for the analysis.

6.2.8.2 New bone area and contact with the implant

The Kruskal-Wallis Test for non-parametric data was carried out to examine any differences between treatment groups. Significance was established as $p \leq 0.05$. If a significant difference was found, a post-hoc, pair-wise Mann-Whitney Test was used to examine any differences between two treatment groups. The statistical software SPSS (SPSS v10.1, SPSS Inc, Chicago, USA) was used for the analysis.

6.3 RESULTS

6.3.1 GRF analysis

The graph below shows the mean percentage pvGRF (figure 6.9). Mean post-operative pvGRF of the right limb at 12 weeks was expressed as a percentage of mean pre-operative pvGRF of the right limb, (right post operative / right pre-operative x 100), and was greater in the MSC group than the control or the osteoblast group at 108.16% (±10.99 SE), 82.54% (±8.30 SE) and 60.83% (±6.17 SE) respectively. The Kruskal Wallis test showed there was a significant difference between the groups at p=0.036. The post-hoc Mann-Whitney U Test revealed the difference to be significant between the MSC and osteoblast group at p=0.034, however there was no significant difference between any of the other paired groups.

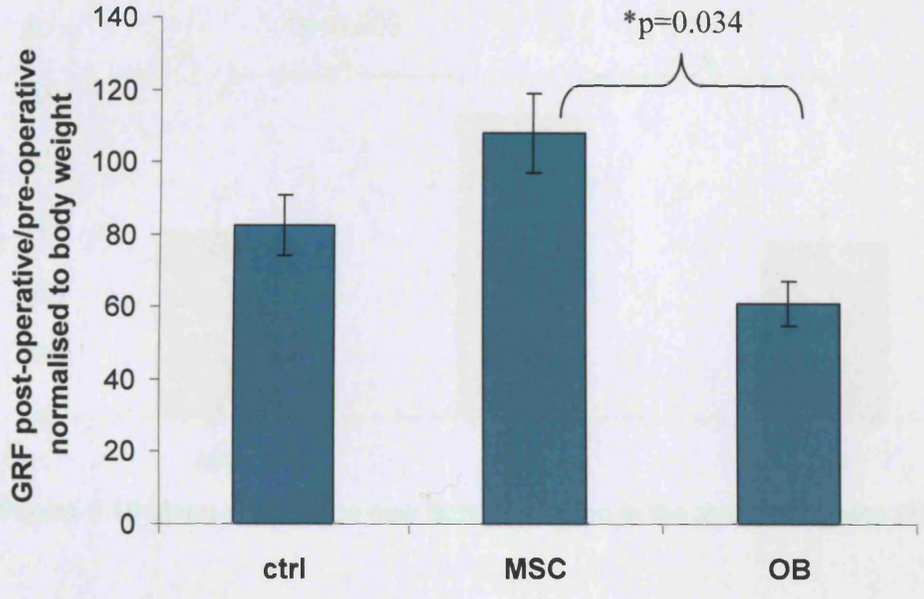


Figure 6.9 Mean percentage pvGRF with post operative values expressed as a percentage of pre-operative values in the control, MSC and osteoblast group (±SE). *Mann-Whitney U Test (p≤0.05).

6.3.2 New Bone Formation

The data for new bone formation was plotted as the mean percentage new bone formation in the available space for each treatment group and is shown in the figure below (figure 6.10). The Kruskal-Wallis Test revealed a significant difference in new bone formation between all the groups (p=0.001). On closer inspection, using the Mann-Whitney U test, there was a significant difference between the amount of new bone in the control, at 28.69% ± 3.9SE and the MSC group, with more new bone in the MSC group at 46.77 % ± 3.27SE (p=0.003). Mann-Whitney U tests also revealed significantly more new bone in the MSC group compared to the osteoblast group which had an average new bone formation of 27.25% ± 2.88SE (p<0.01). However there was no significant difference in new bone formation between the control and the osteoblast group.

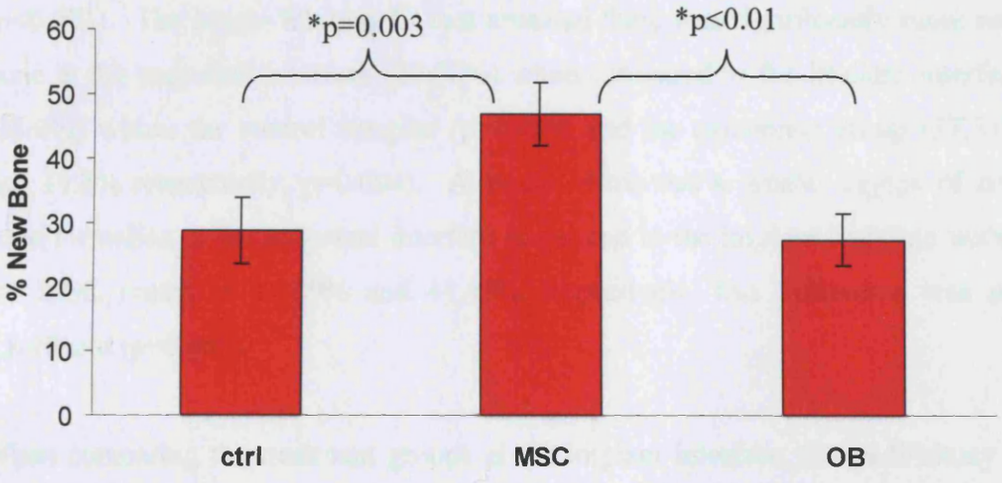


Figure 6.10 Mean percentage new bone formation in the available space (±SE)

6.3.3 New Bone Formation at Implant and Host Interfaces

The data representing mean percentage new bone formation in the available space was separated into the transect analysed at the graft/implant interface termed 'inner' and the transect at the host/graft interface termed 'outer' and plotted in the graph below in order to compare the two interfaces (figure 6.11). There is a trend towards more new bone at the endosteal interface within all the groups with 39.95%, 51.39% and 37.31% in the endosteal region compared with 25.0%, 44.43% and 19.8% in the implant interface for control, MSC and osteoblast groups respectively. Scaffolds with MSCs incorporated into the allograft displayed a higher mean value of new bone than any other treatment group within implant and endosteal interfaces and the osteoblast group produced less new bone within implant and endosteal interfaces than MSC or control groups.

The Kruskal-Wallis Test showed a significant difference between all the groups ($p<0.001$). The Mann-Whitney U Test revealed there was significantly more new bone at the endosteal interface (39.95%) when compared to the implant interface (25.0%) within the control samples ($p=0.039$) and the osteoblast group (37.31% and 19.8% respectively, $p=0.004$). Although there was a greater degree of new bone formation at the endosteal interface compared to the implant interface within the MSC group, at 51.39% and 44.43% respectively, this difference was not significant ($p=0.462$).

When comparing the treatment groups at the implant interface, Mann-Whitney U tests showed that there was significantly more new bone in the MSC treated group at 51.49% compared to the control at 39.95% ($p=0.015$), and in the MSC group at 51.49% than in the osteoblast group at 37.31% ($p<0001$). However, although there was more new bone in the control compared to the osteoblast group, this was not significant ($p=0.743$). When comparing the treatment groups within the endosteal interface, the MSCs had more new bone than the control group at 44.43% and 25.0% respectively, however this was not significant ($p=0.089$). The MSCs also had more new bone than the osteoblast group (19.8%) and the control had more new bone than the osteoblast group, neither of which were significant ($p=0.073$ and $p=0.818$ respectively).

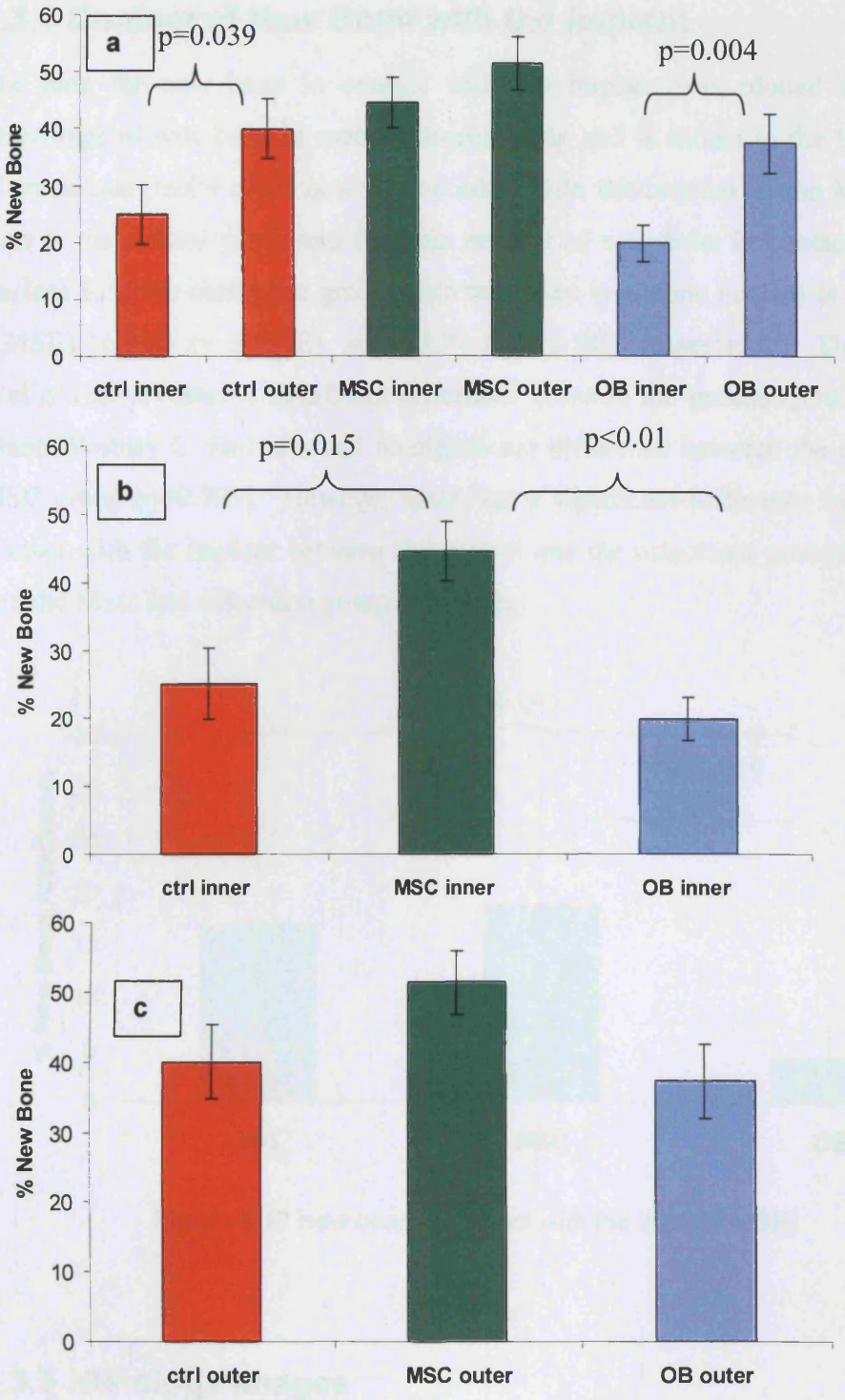


Figure 6.11a-c Percentage New Bone Formation comparing the implant interface ('inner') with the host interface ('outer'): **a** p values show significantly more bone in the outer group within control and osteoblast samples; **b** there is a significant difference between treatments in the inner groups between MSC and control and MSC and osteoblasts; **c** there is no significant difference between treatments in the outer groups.

6.3.4 Contact of New Bone with the Implant

The data for new bone in contact with the implant was plotted as a mean percentage of new bone in each treatment group and is shown in the figure 6.12. There is marginally more new bone contact with the implant in the MSC group than in the control group and the least amount of new bone in contact with the implant is in the osteoblast group, with the mean new bone contact at 18.71% (± 6.31SE) 16.92% (± 6.15SE) and 4.12% (±4.12 SE) respectively. The Kruskal-Wallis Test revealed a significant difference between the groups (p=0.015). The Mann-Whitney U Test revealed no significant difference between the control and MSC group (p=0.775). However there was a significant difference in new bone contact with the implant between the control and the osteoblast groups (p=0.041) and the MSC and osteoblast group (p=0.019).

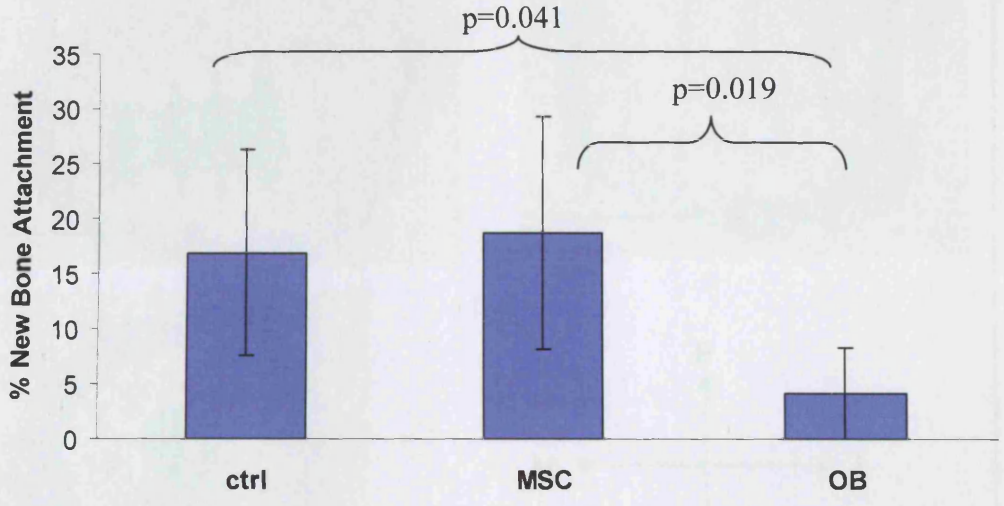


Figure 6.12 New bone in contact with the implant (±SE)

6.3.5 Histology Images

6.3.5.1 Time Zero

Impaction grafting at time zero was performed on cadaveric bone under the same operative conditions as in a living animal. Graft was impacted evenly around the stem of the prosthesis so that the stem was never in contact with the host bone. The images below show that there was always a layer of graft between the stem and the

endosteal cortex (figure 6.13). This shows that the model was appropriate for studying bone induction in impaction grafting. If contact of the implant with the endosteal surface had been allowed, bone may have been recruited directly from the host and osteoinductivity associated with the graft or the cells may have been less influential. Allograft granules were between 2-5mm in size and were of similar distribution along the stem so that small graft did not sink to the tip. Distribution of graft was shown to be even in the cadaveric animal at time zero (figure 6.13). There is evidence of the original trabecular bone remaining in the proximal section (figure 6.13). However, this is in the lesser trochanter and removal of this bone would not equate to a real revision situation.

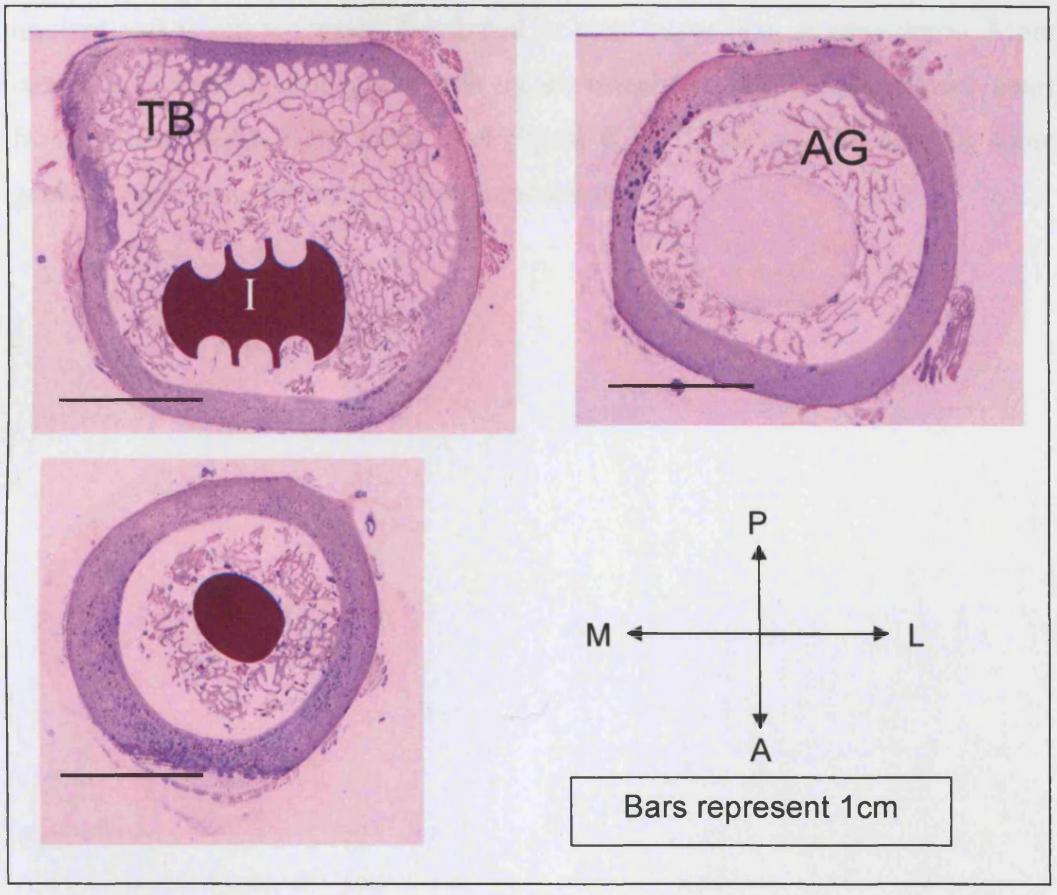


Figure 6.13 Macroscopic images of toluidine blue and Paragon stained transverse sections through a femur at time zero. Images represent Proximal (top left), Mid (top right) and Tip (bottom left) levels. The black line represents 1cm. The arrow directions represent Posterior (P), Medial (M) Lateral (L) and Anterior (A) aspects. 'TB' is trabecular bone, 'AG' is allograft, 'I' is implant. The implant detached from the slide during processing of the Mid section.

6.3.5.2 Histology at 12 weeks

The images below are transverse sections through the femur 12 weeks after impaction grafting surgery in the control, MSC and OB groups (figure 6.14). Graft can be seen adjacent to the implant interface in both the control and MSC sections at the mid level. All other implant surfaces are surrounded by fibrous tissue which appears light pink in the images, or there is a gap between the implant and the organic tissue which is a processing artefact causing the tissue to separate from the implant.

These images show that tissue has filled the gap between the cortex and the implant and is more densely distributed in most cases than at time zero. More bone can be seen in conjunction with the endosteal interface in all instances apart from the osteoblast group at tip level (figure 6.14). This may be because some graft was lost from the section during processing.

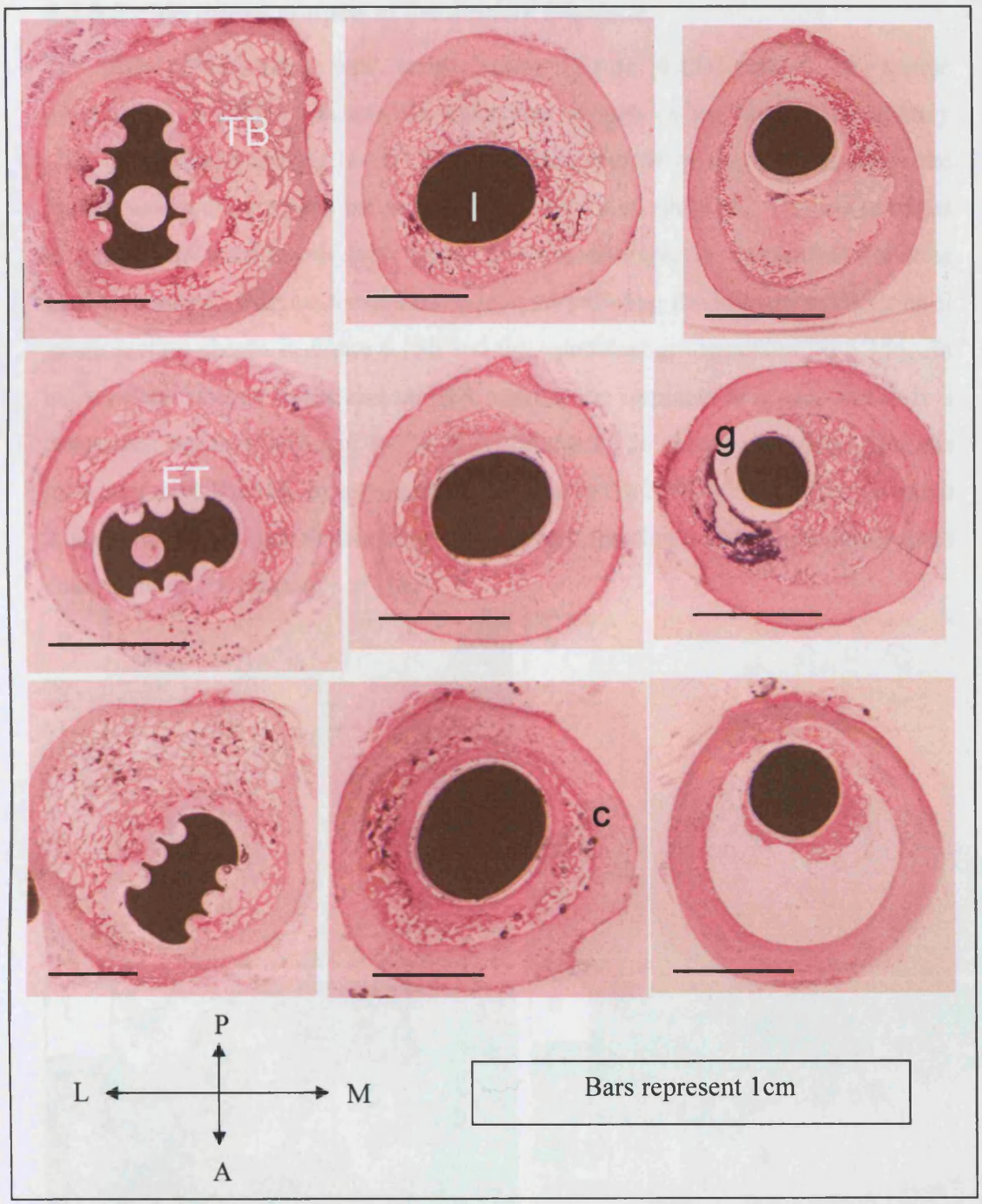


Figure 6.14 Macroscopic histological images of toluidine blue and Paragon stained transverse sections through the femur harvested after 12 weeks *in vivo* at Proximal (left column), Mid (central column) and Tip (right column) levels. The top row is the control group, the middle row is the MSC group and the bottom row is the OB group. 'I' is the implant, 'TB' is trabecular bone, 'FT' is fibrous tissue, 'C' is the cortex, 'g' is the processing artefact surrounding the implant. The black line on each image represents 1cm. The arrow directions represent Posterior (P), Medial (M), Lateral (L) and Anterior (A) aspects.

6.3.5.3 High power images at the implant interface

The high power, time zero image below (figure 6.15a) shows the sparse distribution of allograft across the transverse section of the femur immediately after impaction at the tip level. There is little contact of the allograft with the implant and there are gaps between adjacent pieces of allograft. The rough edges of some of the graft pieces show where the force of impaction has caused the bone graft to fracture. Fibrous tissue can be seen surrounding the implant in the control group section shown in figure 6.15b and the osteoblast group section in 6.15d. In between the fibrous tissue and the HA coating the implants is a gap, which is a processing artefact, adjacent to the HA. Figure 6.15c shows new bone that has integrated with the HA layer coating the implant in the MSC group. The new bone is also contiguous with the surface of the implant forming a virtually uninterrupted coating along the boundary of HA.

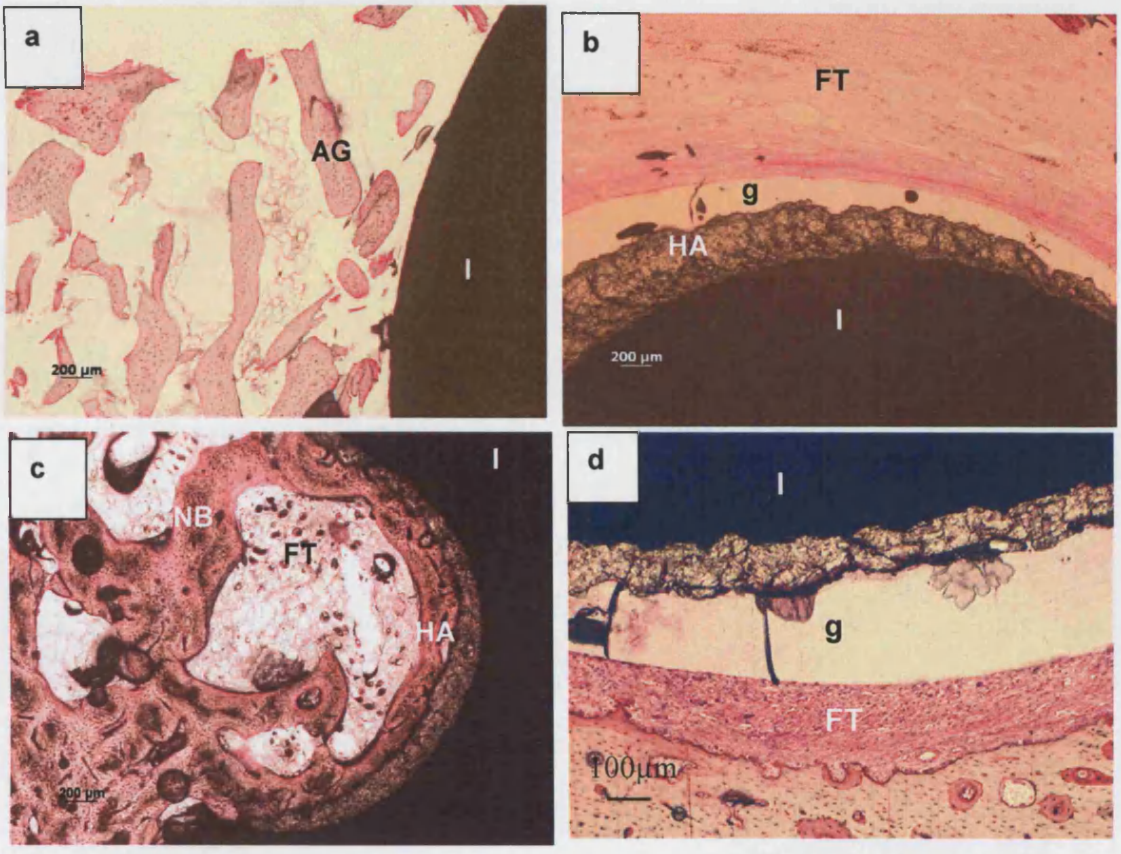


Figure 6.15 Transverse sections of the femur at different levels in the implant interface. **a** Time zero; **b** Control group at 12 weeks; **c** MSC group at 12 weeks; **d** OB group at 12 weeks; 'AG' is allograft, 'I' is the implant, 'HA' is hydroxyapatite, 'g' is an artefact induced gap, 'NB' is new bone, 'FT' is fibrous tissue.

6.3.5.4 Control Group

The images below are typical of samples from an impaction grafted THR control after 12 weeks *in vivo*. Bone formed directly, without endochondral ossification, on existing areas of allograft bone (figure 6.16a). Evidence of new bone formation can be seen in the pink, darkly stained matrix. New bone formed around the edges of fragmented and intact allograft granules (figure 6.16a). Fibrous tissue has formed in some of the spaces between impacted allograft granules in figure 6.16a. Figure 6.16b shows new bone following the contours of the endosteal surface adjacent to the femoral cortex. This is indicative of the majority of new bone arising from osteoinduction derived from host bone and osteoconduction along the host bone. New bone also follows the coastal regions of individual allograft particles in a thin strip showing that the allograft is osteoinductive (figures 6.16b and 6.16c).

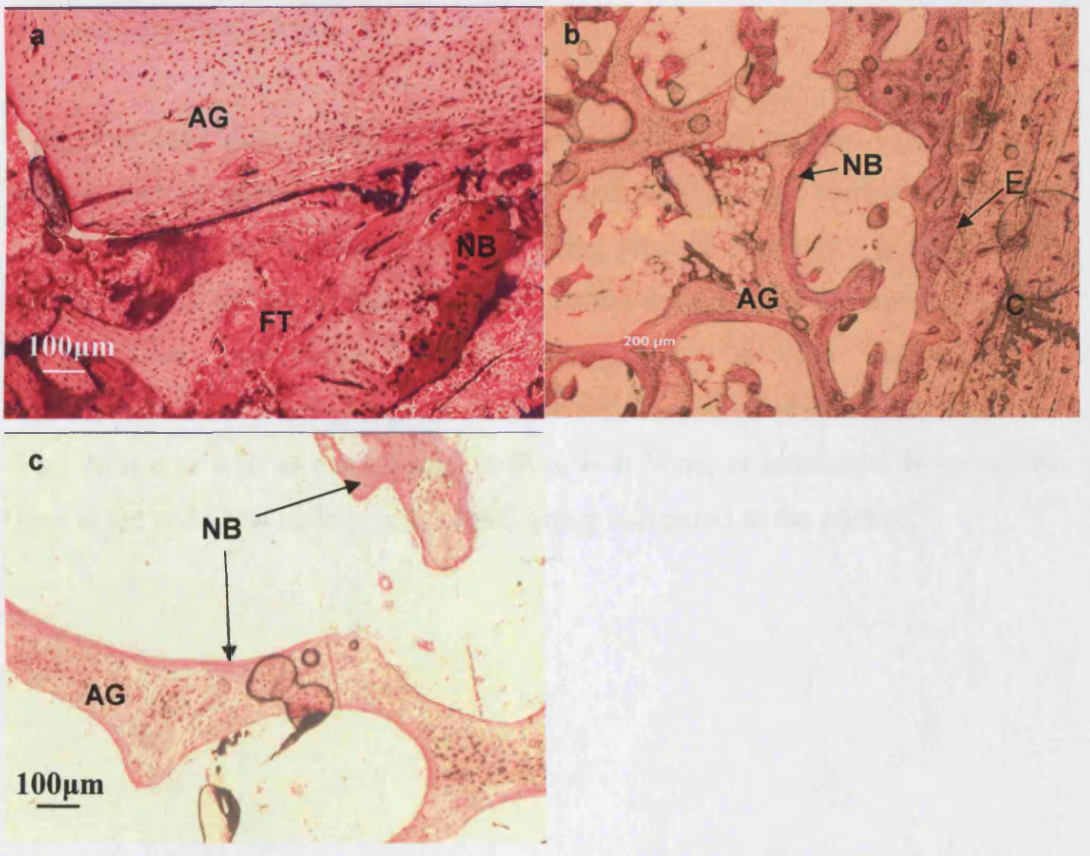


Figure 6.16a-c Typical examples of control samples showing areas of allograft (AG), fibrous tissue (FT) and new bone (NB), **b** Osteoconduction and osteoinduction of new bone along the endosteal surface (E), showing the cortex (C), **c** New bone following the contours of the allograft granules.

6.3.5.5 MSC Implant

The images below are typical of samples from an impaction grafted THR using allograft seeded with MSCs. After 12 weeks *in vivo,* surfaces of new bone were covered in a layer of osteoblasts, and surrounded by unmineralised osteoid. Osteoblasts can be clearly distinguished from the mesenchyme and blood cells by their plump, basophilic morphology and are mostly encased by the osteoid. The osteoblasts are providing new bone in direct intramembranous ossification (figure 6.17a).

There is no evidence of allograft in figure 6.17a or 6.17b, however new bone is mostly seen to be associated with remaining allograft in this study. Although it was observed that a large proportion of the bone in this group formed by intramembranous ossification, figure 6.17c shows evidence of bone formed by endochondral ossification.

Large cartilage lacunae can be observed on the allograft surface. There is also evidence of intramembranous ossification where darkly purple stained osteoid has formed without any underlying allograft being present. However, the allograft on which the osteoid has formed could be outside the plane of the image. Figure 6.17d shows new bone following the contours of the endosteum by osteoconduction. In this group it is possible that the source of new bone is derived from MSCs as well as osteoinduction from host bone, as more new bone can be seen at the endosteal surface in the MSC group compared to the control.

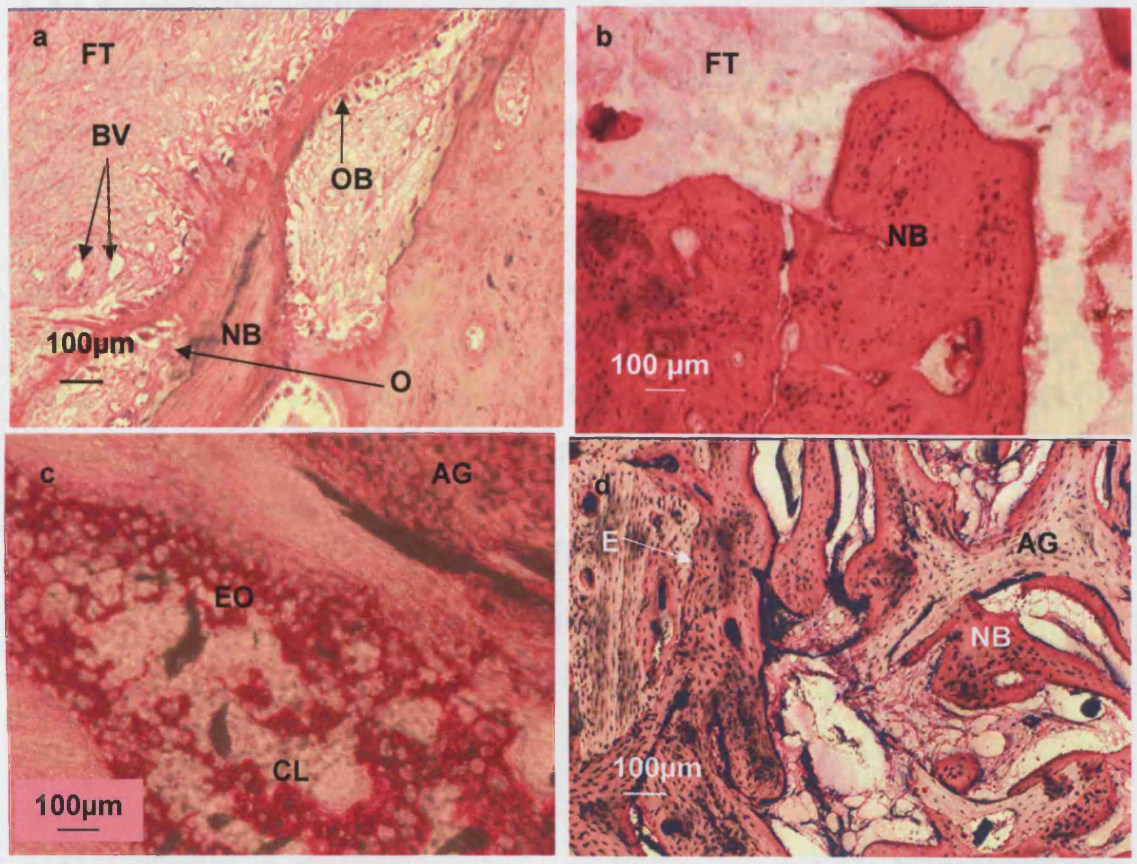


Figure 6.17a-d Sections through the middle of an MSC / allograft impacted THR after 12 weeks *in vivo*: **a** There is evidence of new bone (NB) and fibrous tissue (FT), osteoblasts, osteoid (O), blood vessels (BV); **b** New bone and fibrous tissue; **c** Endochondral ossification (EO) on allograft surface indicated by cartilage lacunae (CL); **d** New bone at the endosteal surface (E).

6.3.5.6 Osteoblast Implant

In the allograft seeded with osteoblasts group, new bone is formed in a similar manner to the control and MSC treated groups. New bone surrounds granules of remaining allograft and it is possible that new bone may have formed without association with allograft as some new bone appears to be surrounded by fibrous tissue (figure 6.18). New bone is not always associated with allograft in the osteoblast group as in the MSC group. Most of the new bone in the osteoblast group is associated with the endosteum (figure 6.18b). Some of the allograft granules appeared to have relatively little new bone attached to them compared to the control and the MSC group.

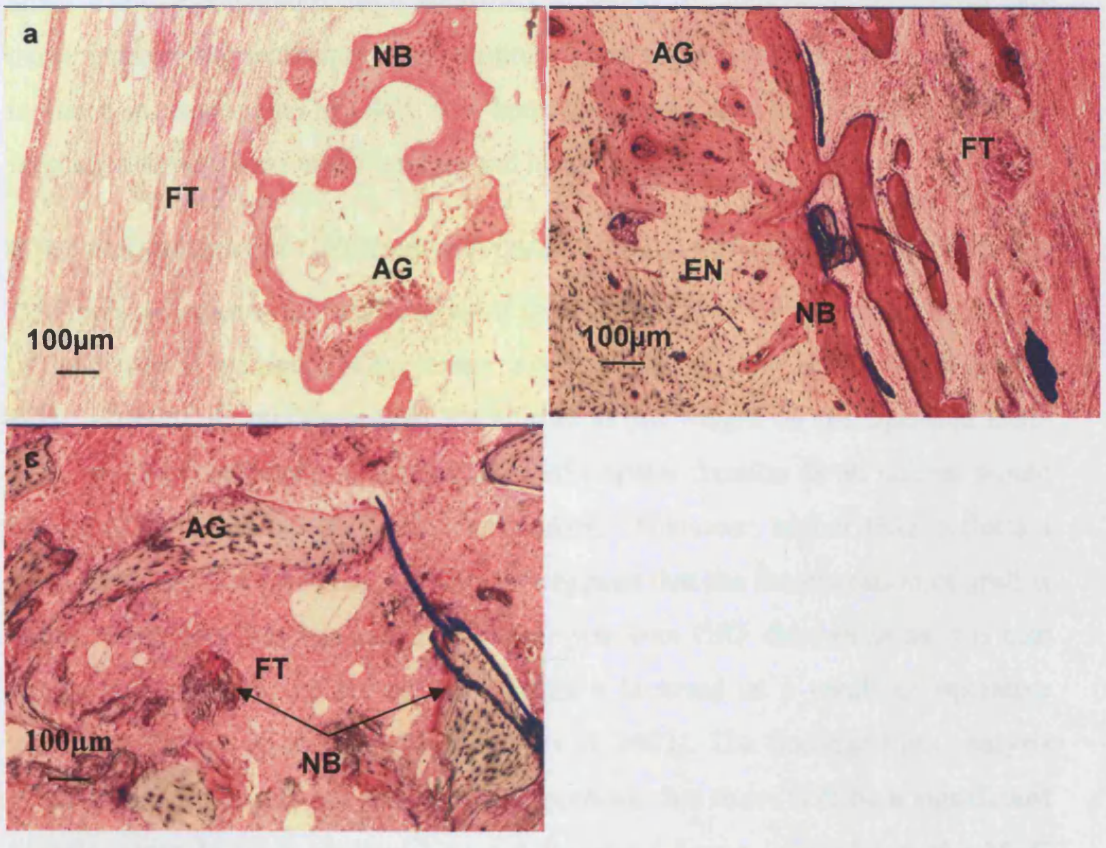


Figure 6.18a-c Sections through the middle of an osteoblast treated allograft impacted THR; **a** There is evidence of allograft granules 'AG', fibrous tissue 'FT' and new bone 'NB' which is associated with and independent of existing allograft; **b** New bone attached to the endosteum 'EN'; **c** allograft granules and new bone.

6.4 DISCUSSION

The aim of the study in this chapter was to improve new bone formation in an allograft impaction THR model in sheep by the addition of MSCs or MSC derived osteoblasts with allograft as a control. The study was required because bone was impacted under realistic conditions. New bone formation would have been associated with the local mechanics of the intramedullary cavity in THRs; also the local factors influencing bone formation were different to those in the ectopic study in Chapter 5 and included osteoinduction and osteoconduction from the host bone. Various parameters were measured to assess the degree of success of this tissue engineering technique to reconstitute bone stock in the sheep femur. This included measurements of GRF, new bone formation, new bone contact with the implant, and new bone at the implant and host interfaces.

6.4.1 Comparison of GRF between control, MSC and osteoblast groups

GRF was an indicator of functionality of the operated limb as it gave an expression of the degree of the return of the femur to normal use after the THR. The degree of GRF relates to the willingness of the animal to put weight on the operated limb. This correlates indirectly with the quality of implant fixation as an animal would be less likely to put weight on a loose implant. Therefore, higher GRF reflects a more adequate fixation of the implant and suggests that the incorporation of graft is better. Definitive conclusions cannot be drawn from GRF data alone as it is also related to the degree of muscle manipulation incurred as a result of operative technique (Bassey *et al.* 1997; Bergmann *et al.* 2001). The findings from analysis of GRF in this study do not support the hypothesis that **there will be a significant improvement in peak vertical Ground Reaction Force (pvGRF) in the MSC group compared to the control and osteoblast groups.** However, the magnitude of GRF was greatest in the MSC group, less in the control group and least in the osteoblast group. The osteoblast group produced significantly less GRF than the MSC group. This is evidence that the use of osteoblasts may have no effect on the function of the limb. It does not demonstrate that the application of osteoblasts is necessarily detrimental to limb function as the GRF is not significantly less in the osteoblast group than in the control group. The hierarchy of MSC superiority over

the control, which is better than osteoblast cells in terms of limb function, is also observed in new bone formation.

6.4.2 The effect of cells on new bone formation

The order of magnitude of new bone formation was greatest in the MSC group, then the control group and finally the osteoblast group. This was the same trend as that of the GRF data. **The hypothesis that: there will be a significant increase in new bone formation in the MSC group compared to the control and osteoblast group can be accepted** because there was significantly more new bone in the MSC group than in the control. The MSC group also produced significantly more new bone than the osteoblast group. MSCs had been differentiated and identified as osteoblasts by their increased production of ALP in chapter 3, and mineralisation. In this chapter, as in chapter 5, the trend is also for more new bone in the MSC treated group than in the control or the osteoblast group. There is also some evidence in the literature that the effect of adding osteogenic media to MSCs cultured on scaffolds does not enhance new bone formation compared to untreated cells on scaffolds (Kruyt *et al.* 2004a). MSCs were pre-differentiated because, as discussed in chapter 1, it is possible that osteogenic commitment *in vitro*, prior to implantation, may provide more rapid and consistent bone formation (Caplan A.I., 2000). However, the data for new bone formation in this chapter suggests that it is more beneficial to transplant a heterogeneous population of primitive MSCs that would already be interacting via cell-cell communication in order to adapt better to the *in vivo* environment.

6.4.3 New bone formation at implant and host interfaces

Evaluation of new bone at the interface of allograft with the implant and with host bone was made by comparing both interfaces within each group. There was a trend of more new bone produced at the host interface in all the groups. **The hypothesis that: there will be a significant increase in new bone formation at the host allograft interface compared with the host implant interface was not accepted.** Both the control group and the osteoblast group produced significantly more new bone at the host interface than at the implant interface. However, although more new bone was found at the host interface than at the implant

interface in the MSC group, there was no significant difference between these regions. The source of new bone could be osteoconduction from inductive factors such as BMPs and MSCs or osteoblasts residing in host bone. One explanation of these results is that diffusion of nutrients, cells and vascularisation towards the implant interface reduces with distance from the endosteal interface, thereby decreasing the contribution of new bone from the host. The control reflects this principle. My study indicates that new bone is derived, either directly or indirectly, from implanted MSCs. These are distributed evenly throughout the allograft before implantation and in this study application of these cells resulted in a more even distribution of bone formation, indicating that MSCs make a contribution to bone formation at the implant interface. According to data for new bone formation, there is not a large contribution of new bone from osteoblasts as they produce the least amount of new bone. The more mature cells such as osteoblast cells are known to have a higher metabolic activity and therefore require greater oxygen supply (Vacanti 2004). This could diminish their ability to survive in a low oxygen environment such as a large graft, where nutrient diffusion would be slow in some areas especially at the implant interface.

The trend whereby more new bone was produced in the MSC group was observed when respective interfaces from each group were compared. **The hypothesis that: there will be a significant increase in new bone formation in the MSC group compared to the control and osteoblast groups within the allograft implant interface or the host allograft interface was not accepted.** The fact that there was significantly more new bone in the MSC group compared to the control group at the implant interface, but no significance in the greater amount in the MSC group at the host interface, shows that the MSCs made more of a contribution to new bone formation at the implant interface than at the host interface. The same is true of the significantly greater proportion of new bone at the implant interface in the MSC group compared to the osteoblast group, whereas at the host interface the increase in the MSC group over the osteoblast group is not significant. There was more bone in the control group than in the osteoblast group at both interfaces. However, this was not significant at either interface, indicating that osteoblast cells are not effective in creating more new bone at interfaces, but neither are they

detrimental to bone formation where analysis is limited to the amount of new bone at implant or host interfaces.

6.4.4 Contact of new bone with the implant

The order of magnitude of contact with the implant in descending order was the MSC group, then the control group and finally the osteoblast group. This trend followed that of new bone formation and GRF data. **The hypothesis that: there will be a significant increase in contact of new bone with the implant in the MSC group compared to the control and the osteoblast group was not accepted.** The amount of new bone contact was only slightly greater in the MSC group compared to the control, and was not significant. However, in this instance there was significantly less new bone in the osteoblast group than in the MSC and control group. Contact with the implant implies that the new bone is more likely to be attached to the implant and will therefore be well integrated for greater stability. The fact that there was significantly less contact with the implant in the osteoblast group indicates that osteoblast cells may be detrimental for bone contact and potentially, stability of the implant. The explanation for this may be that introduction of osteoblasts triggers osteoclastogenesis via the RANK pathway as osteoblasts cells have receptors for osteoclast precursors (Khosla 2001). This may have sped up the cycle of resorbtion of new bone at the implant surface.

6.4.5 Experimental design

The ovine hip model was equivalent to a revision hip replacement and the procedures followed were similar to those used in a clinical impaction grafting. The sheep were skeletally mature and over 2 years old. They were all of a similar age as observed by the fact that the sheep were chosen on the basis of retention of all of their teeth. The duration of the study was 12 weeks. The length of the study was the same as in chapter 4 and has already been discussed in the context of other studies cited in the literature (see section 4.4.3). MSC and osteoblast cells were seeded in greater numbers than in the ectopic study in chapter 4. The numbers of cells were increased from 6 x 10^6 cells/cm^3, in the ectopic study, to 10 x 10^6 cells/cm^3 in this chapter and were more representative of the quantity employed in other studies in the literature where the seeding of cells has been effective in

encouraging new bone formation (see section 4.4.4.). A study by Brewster confirmed, by analysing the stress/strain behaviour of morsellised cancellous bone graft in a Jenike shear box, that graded graft improved the mechanical strength of the morsellised bone. The drawback of graded graft is that close packing may inhibit the revascularisation and therefore incorporation of the graft. Grading the graft to test the theory *in vivo* is beyond the remit of this thesis, therefore the graft will be sieved to the size commonly used in clinical practice. Gie, G.A. *et al.* stipulates that morsellised graft used in a revision THR should be large enough to allow the compressed chips to have 'body'. This excludes bone slurry as the consistency is too low (Gie *et al.* 1993). A case study of four patients summarised the technique commonly used in impaction grafting and morsellised fresh frozen allograft to between 3 and 5mm in diameter (Nelissen *et al.* 1995). Allograft was used as it is currently more accepted in a clinical setting. Also, allograft was found to encourage more contact of new bone with the scaffold surface in chapter 5. This may be beneficial for attachment and therefore integration of the implant with new bone, thereby increasing the stability of the implant. Scaglione, S. *et al* investigated whether ovine MSCs seeded directly onto a 3D scaffold produced more bone than MSCs cultured in 2D prior to scaffold loading and then implanted ectopically into nude mice. Analysis of new bone was quantified by the amount of bone in the available space, which was the same method of analysis employed in my thesis. The amount of new bone was greater in the 3D seeded constructs than the 2D constructs after 8 weeks. The amount of bone produced after 14 days of static loading in 3D and 2D culture and 8 weeks *in vivo* was 12 % and 6% respectively (Scaglione *et al.* 2005). The percentage of bone produced in Scaglione *et al's* study was less in all groups than that produced in all groups in my study. This may be because the work of Scaglione is in an ectopic site and therefore there is no contribution from osteoconduction. In a study by Kruyt *et al.*, the amount of new bone in the available pore space produced in iliac crest orthotopic sites, where unimpacted BMSC loaded implants were analysed after 12 weeks, was 29.0% compared to 46.77 % in the MSC group in this chapter. The control group produced 25.1% compared to the 28.69% in my study. The discrepancies between these studies are that the defect is considerably smaller in Kruyt's work measuring Ø17 x 6mm (Kruyt *et al.* 2004c). As discussed in Section 4.4.4, the ectopic study by Kruyt shows that BMSCs treated with supplements for

osteogenic differentiation produce a similar amount of new bone on scaffolds (11.2%) as those without supplements (11.7%), which concurs with the findings in this chapter as osteoblast cells do not make a contribution to bone formation. In a study by Bruder *et al.*, MSCs loaded onto ceramic cylinders of Ø14mm x 21mm and placed into canine segmental bone defects created new bone more closely approaching the amounts achieved in this chapter. However the evaluation was carried out after 16 weeks. The mean values for new bone were 39.9% in the MSC treated group and 24% in the control group (Bruder *et al.* 1998b). In Arinzeh's study, where MSCs were also loaded onto a ceramic cylinder for reimplantation into a canine segmental defect and analysed after 16 weeks, there was a comparable amount of new bone produced as in other studies mentioned above, with new bone at 42.3% in the MSC treated group and 24.6% in the control group without MSCs (Arinzeh *et al.* 2003). These percentages are closest to those found in this chapter. The largest discrepancy between the cell seeded and control group was found in Kon *et al's* study, where MSCs were loaded onto HA cylinders and implanted into sheep tibial defects for two months, giving 54.2% new bone compared to controls containing 8.6% new bone.

6.5 CONCLUSION

MSCs added to allograft produce more new bone than either allograft alone or allograft supplemented with osteoblasts. This indicates that MSCs could be used in conjunction with impaction allografting in the clinic. MSC derived osteoblasts make no contribution to new bone formation.

CHAPTER 7: GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

Osteolysis around THRs often results in subsidence and failure of the implant. The loss of bone stock compromises immediate fixation and long term stability of the rTHR. The overall hypothesis for this thesis was that **incorporating MSCs into allograft or hydroxyapatite granules which are then impacted, will increase the rate and extent of bone formation.** The flow diagram (figure 7.1) summarises the questions and conclusions from each Chapter of my thesis that led to answering this overall hypothesis.

It was essential to ascertain whether MSCs survived normal impaction forces used in clinical impaction grafting. Chapter two of my thesis tested the viability of MSCs seeded onto the allograft and impacted at a range of forces obtained during rTHRs. The forces were replicated *in vitro* using an impactor designed by Kirsty Phipps (Phipps 2004). MSCs survived impaction forces up to 6KN. This was within the range of normal impaction forces imparted during surgery. Although MSCs do not survive at 9kN it was shown that mean forces above 6kN impaction were at the highest end of the range and only experienced in a small number of cases (Phipps 2004). It is therefore feasible that impaction can be used without affecting MSCs. Although force was measured, strain may have been an alternative parameter to replicate. However strain is the deformation of a physical body under the action of applied forces. Strain differs depending on where the contact is on each individual granule, so impaction of the granule was measured by graft height as a consequence of the applied force and not stress or resultant strain as this would have been very difficult to measure.

MSCs can be expanded prior to differentiation into osteoblasts, possibly expediting the rate and extent of bone formation compared to MSCs. The question asked was would the osteoblasts survive better than MSCs after impaction on allograft? Chapter three of my thesis tested the viability *in vitro* of osteoblasts derived from MSCs which were seeded onto the allograft and impacted at a range of forces obtained during rTHRs. This study found that osteoblasts can survive impaction forces up to 3kN, which is in the lower range of normal impaction forces used during revision THRs, however these cells did not seem to be as resilient as MSCs.

The greater susceptibility of the osteoblasts to impaction forces may be explained by their involvement in mechano-transduction during bone remodelling. It is not known exactly how bone perceives mechanical stimuli. However the literature suggests that osteoblasts and osteocytes detect strain which can stimulate apoptosis in immature osteoblasts and induce proliferation in older osteoblasts (Weyts *et al.* 2003). In this Chapter osteoblasts were selected after 14 days of differentiation in osteogenic supplements. It is possible that at other stages in the osteogenic lineage, osteoprogenitor cells may respond differently to impaction forces. Further experiments could test the effect of impaction forces on cells at stages phenotypically defined by the temporal expression of cell surface markers on: osteoprogenitor cells, pre-osteoblasts, transitory osteoblasts, secretory osteoblasts, osteocytic osteoblasts and osteocytes (Bruder and Caplan 1989; Bruder and Caplan 1990a; Bruder and Caplan 1990b).

Another avenue for future work is to consider the possibility that MSCs are not the only source of bone forming cells. Mature cells of other tissue types, given the correct stimuli, could differentiate into bone tissue. The controversial theory that a cell from one tissue can transform into another tissue type is termed stem cell 'plasticity'. There are several possible mechanisms of plasticity such as: de-differentiation (also known as retrodifferentiation) where a differentiated cell; converts to a precursor cell or stem cell; trans-differentiation, where a differentiated cell converts to a different type of differentiated cell or transdifferntiation via de-differentiation followed by re-differentiation (Lackshmipathy and Verfaillie 2005). The controversy stems from the fact that assessment of plasticity is not functional but characterised by phenotype and therefore mistakenly attributed to transferal of mixed populations of donor cells. Cell fusion, which is a combination of two or more individual cells forming one cell, can also erroneously be identified as plasticity. Also, a failure to replicate findings adds to the scepticism surrounding plasticity (Lackshmipathy and Verfaillie 2005). However, if plasticity does occur and osteoblasts could be derived from any tissue, then non-invasive procedures could be used to harvest cells such as fibroblasts for tissue engineering of bone. One study shows that when MSC implants become infiltrated with fibroblasts *in vivo*, bone formation is reduced. *In vitro* assays included ALP, calcium and cartilage cultures with different ratios of

human MSCs and fibroblasts and a functional *in vivo* ceramic cube assay in SCID mice for bone formation. In both the *in vitro* and the *in vivo* assays bone formation decreases with increasing numbers of fibroblasts (Lennon *et al.* 2000). Although stem cell plasticity is not investigated in this study, it would suggest that plasticity is not occurring as fibroblasts do not appear to contribute to new bone. However, in fibroblast cultures treated with osteogenic media there was minimal mineralisation and a change in morphology. It could be argued that implanting the cells subcutaneously did not give the fibroblasts the correct environmental cues to differentiate into osteoblasts. An alternative study could involve probing the fibroblasts for osteogenic cell surface markers and implanting fibroblasts into orthotopic sites.

Another approach to tissue engineering would be the co-culture of cells in order to enhance MSC numbers, allowing scale-up for large defects requiring larger numbers of autologous cells or for allogenic widespread clinical application. A study hypothesised that replating non-adherent MSCs that are usually discarded during cell culture would result in more osteogenic MSCs than from primary cultures alone (Wan *et al.* 2006). The authors confirmed that the number of MSCs increased by 36.6% and after subcutaneous implantation in SCID mice on HA/TCP, there was new bone in all implants with MSCs. However, it should be noted that there was no quantative evaluation so it could not be established whether replated cells enhanced *in vivo* bone production (Wan *et al.* 2006). In another study non-adherent osteoblast accessory cells (OAC), which comprise another subset of cells in the heterogenous stem cell compartment, trigger MSC development into the osteogenic lineage stimulating bone precursor cells to express osteonectin and ALP (Eipers *et al.* 2000). In addition, the work by Baksh *et al.* shows increased numbers of osteoblast colony forming units (cfu-o) and cfu-fs from suspension-derived cells of unsorted compared to sorted HSCs and MSCs. Therefore MSC growth is increased by coculture with HSCs (Baksh *et al.* 2003; Baksh *et al.* 2005). A study by Baksh, D. et al has the added advantage of being in serum free culture which is required for clinical grade application of stem cells, although it should be noted that the addition of growth factors not normally used in static culture, were required (Baksh *et al.* 2003). It may be advantageous to grow

MSCs in suspension cultures with HSCs to harvest greater numbers of MSCs with enhanced osteogenic potential by using a similar bioreactor system.

Chapter four of my thesis assessed whether new bone formation of impacted allograft and hydroxyapatite in a sheep critical sized defect was improved by using autologous sheep MSCs *in vivo.* In addition osteoconductivity of irradiated allograft was compared with 60 percent porous granular HA in an animal model that simulates bone remodelling within impacted graft *in vivo.* There was no significant increase in new bone formation or remodelling with the introduction of MSCs on impacted allograft or HA granules. Both scaffolds were shown to induce similar amounts of new bone formation, although there was a significantly greater degree of resorption of the allograft allowing more ingrowth of fibrous tissue. In a femoral defect model, 'controlled compression is applied over the defect area, whereas grafting material impacted around a femoral stem is exposed to a combination of compression and shear forces' (Griffon *et al.* 2001). No other study has attempted to implant MSCs at the femoral condyle. The size of the graft and interfragmentary strain could have prevented penetration of blood vessels and transport of nutrients to the cells, reducing new bone formation. The literature shows that MSCs contribute to new bone formation *in vivo* in bony sites (Arinzeh *et al.* 2003; Kadiyala and Jaiswal 1997; Bruder *et al.* 1998b). Based on this data it is reasonable to postulate that the distribution of mechanical forces at the bone ends may have had a detrimental effect on new bone formation. In contrast, although MSCs did not increase new bone formation it was possible that the model was very osteoinductive due to the large quantities of healthy trabecular bone surrounding the allograft / cell composite. The cells from the host may have masked the contribution of new bone from implanted MSCs. Considering this explanation, it would still be feasible to use MSCs in a revision hip replacement where the surrounding tissue has a compromised vasculature or an ectopic situation where the surrounding tissue is not osteoinductive, in order to test the osteoinductive capability of the cells. Future work could include increasing the number of cells used in this study to test for the osteoinductive capacity of MSCs and restricting the micromovement of the graft particles by sealing the defect with gauze.

Chapter five of my thesis investigated whether MSCs or osteoblasts contribute to bone formation in impacted allograft or an allograft and HA combination in an ectopic site. In all cases there is an increase in new bone formation when MSCs are used. The use of osteoblasts did not enhance the amount of new bone on any scaffold. This study has shown that MSCs increased the production of new bone formation on impacted allograft and in an impacted HA/allograft. There was a significant increase in new bone in the impacted HA/allograft and unimpacted block HA group with MSCs. However, there was no significant difference between the control and OB groups. Therefore this study showed that in an ectopic site MSCs had a significant positive effect.

Chapter 5 also showed that allograft produces the most new bone in contact with its surface compared to all the other scaffolds, although there is no significant difference in new bone contact between the scaffolds. The MSCs also produce more new bone in contact with the scaffold surface than any other treatment group. Surgeons should consider using allograft in combination with MSCs, in preference to HA and allograft combined, as this combination encourages osteoconduction which may lead bone to integrate with the implant surface. Pull-out tests of MSC treated, allograft impacted hemiarthroplasties would confirm the stability of the implants. Future work could involve the addition of osteoblasts on an impacted HA scaffold to see if they encourage further osteogenic differentiation and therefore more new bone.

Chapter 6 of my thesis aimed to regenerate new bone in defect areas adjacent to revision hip replacements using impaction grafting with allogenic bone combined with either MSCs or osteoblasts derived from MSCs. MSCs produce more new bone than the control group which produces more new bone than the osteoblast group. In the case of new bone, the difference is significant between the MSC and control group. MSC derived osteoblasts make no contribution to new bone formation and have a detrimental effect on new bone contact with the implant in this impaction model. HA or an HA and allograft mix with MSCs or osteoblasts could have been additional groups used in this part of the study in Chapter 6. In Chapter 5 HA combined with MSCs induced the most ectopic bone. However concern over the stability and strength of HA in an impaction grafting situation led

me to use allograft. Cement was not used in the hemiarthroplasties in Chapter 6 due to the concern that MSCs would have died when subjected to the temperature of cement during polymerisation. There is a possibility that micromotion may be increased in uncemented stems. If cement had been used, stability of the implant may have allowed new bone produced by MSCs to attach to the implant. Further studies could include analysis at later time-points, such as 6 months or even longer, to provide insight into the pattern of bone turnover and whether new bone formation was maintained long term *in vivo*. In addition osteointegration of new bone with the implant could have been tracked over time. Tracking the MSCs in the *in vivo* experiments by GFP or Lac Z labelling and subsequently investigating organs for these markers may indicate whether MSCs have contributed directly to new bone formation at the defect site or systemically migrated to other tissues. The next stage of investigation should also involve validating the function of new bone by using a pull-out test. In addition, density scanning using DEXA (Dual Energy X-Ray Absorptiometry) could analyse real time quality and remodelling of new bone.

7.2 CONCLUSIONS

- MSCs survive normal impaction forces used in clinical impaction grafting in the range of 3-6kN.
- Osteoblasts derived from MSCs survive normal impaction forces used in clinical impaction grafting at 3kN only and are therefore more susceptible to impaction.
- There was no significant increase in new bone formation or remodelling with the introduction of MSCs on impacted allograft or HA granules in the femoral defect model.
- There was a significantly greater degree of resorption of the allograft allowing more ingrowth of fibrous tissue in the femoral defect model.
- MSCs increase new bone on any scaffold after impaction in an ectopic site with a significant increase in the HA/allograft and unimpacted block HA group with MSCs.
- There is no advantage of seeding osteoblasts on graft as there is no significant difference between control and osteoblast seeded scaffolds in an ectopic site.
- MSCs contribute to new bone formation in the rTHR model and this is especially evident in regions where new bone formation is compromised such as regions adjacent to the implant interface.

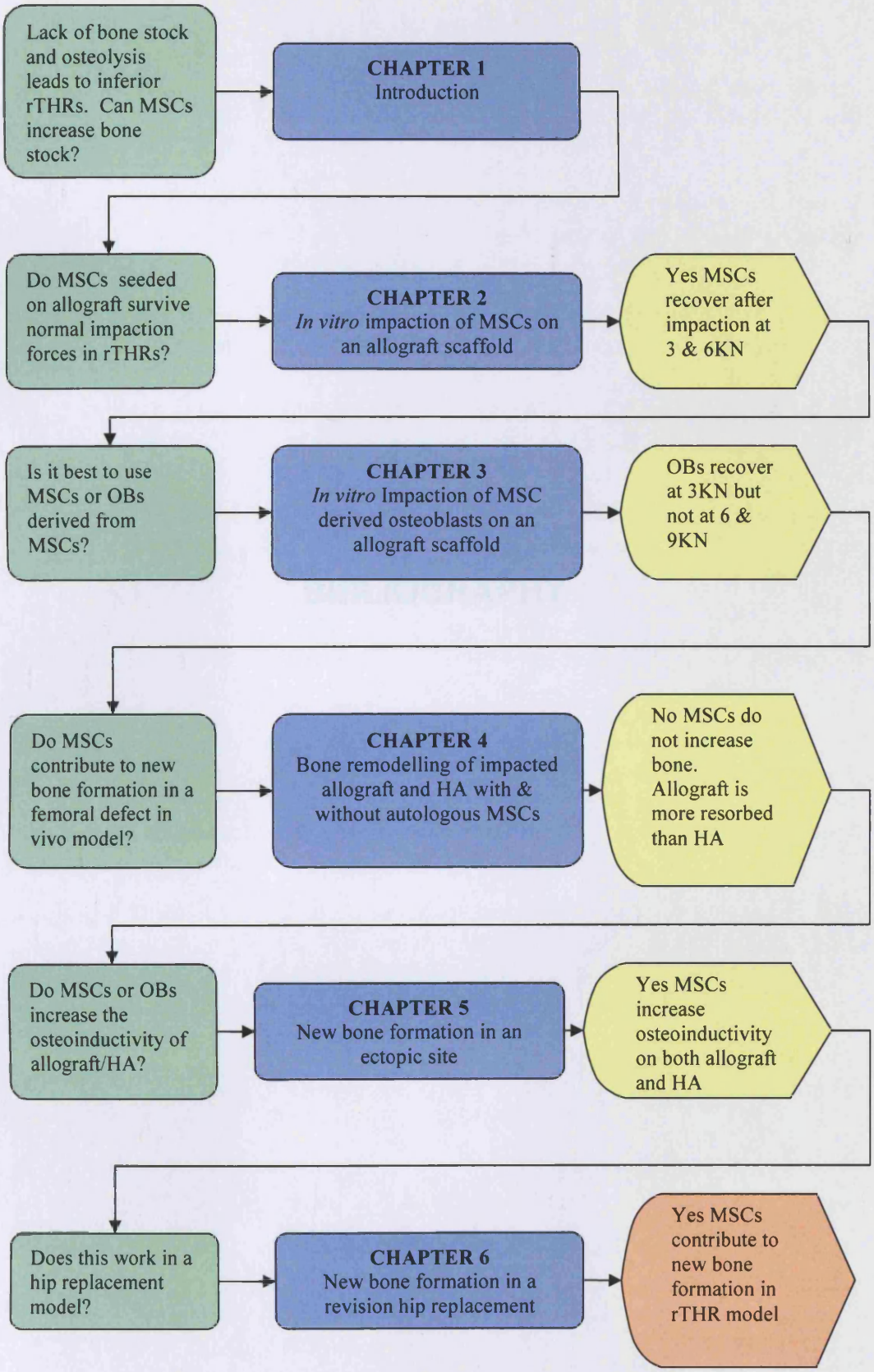


Figure 7.1 Thesis Flow Diagram

BIBLIOGRAPHY

Abu, E. O., Horner, A., Kusec, V., Triffitt, J. T., and Compston, J. E. (2000) The localization of the functional glucocorticoid receptor alpha in human bone. *J Clin Endocrinol Metab* 85, 883-889.

Aerssens, J., Boonen, S., Lowet, G., and Dequeker, J. (1997) Interspecies differences in bone composition, density, and quality: potential implications for *in vitro* bone research. *Endocrinology* 139, 663-670.

Amstutz, H. C., Campbell, P., Kossovsky, N., and Clarke, I. C. (1992) Mechanism and clinical significance of wear debris-induced osteolysis. *Clin Orthop Relat Res* 7-18.

Andrew, C. and Bassett, L. (1972) Clinical implications of cell function in bone grafting. *Clin Orthop Relat Res* 87, 49-59.

Arinzeh, T. L., Peter, S. J., Archambault, M. P., Van Den Bos, C., Gordon, S., Kraus, K., Smith, A., and Kadiyala, S. (2003) Allogenic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *J Bone Joint Surg* 85-A, 1927-1935.

Athanasou, N. A. (1996) Cellular biology of bone-resorbing cells. *J Bone Joint Surg* 78-A, 1096-1112.

Bachoud-Levi, A.-C., Gaura, V., Brugières, P., Lefaucheur, J.-P., Boissé, M.-F., Maison, P., Baudic, S., Ribeiro, M.-J., Bourdet, C., remy, P., Cesaro, P., Hantraye, P., and Peschanski, M. (2006) Effect of fetal neural transplants in patients with Huntington's disease 6 years after surgery: a long term follow up. *Lancet Neurol* 5, 303-309.

Baksh, D., Davies, J. E., and Zandstra, W. (2005) Soluble factor cross-talk between human bone marrow-derived hematopoietic and mesenchymal stem cells enhances in vitro CFU-F and CFU-O growth and reveals heterogenity in the mesenchymal progenitor cell compartment. *Blood* 106, 3012-3019.

Baksh, D., Davies, J. E., and Zannettino, A. (2003) Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion independent survival and expansion. *Exp Hematol* 31, 723-732.

Baksh, D., Song, L., and Tuan, R. S. (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 8, 301-316.

Barbash, I. M., Chouraqui, P., Baron, J., Feinberg, M. S., Etzion, S., Tessone, A., Miller, L., Guetta, E., Zipori, D., Kedes, L. H., Kloner, R. A., and Leor, J. (2003) Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium. *Circulation* 2003, 863-868.

Bassey, E. J., Littlewood, J. J., and Taylor, S. J. G. (1997) Relations between compressive axial forces in an instrumented massive femoral implant, ground

reaction forces, and integrated electromyographs from vastus lateralis during various osteogenic exercises. *J Biomech* 30, 213-223.

Bell, E. (2000) Tissue Engineering in Perspective. In: **Principles of Tissue Engineering,** pp. xxxv-xli. Eds R. P. Lanza, R. Langer, J. Vacanti. Academic Press: London.

Beresford, J. N. (1989) Osteogenic stem cells and the stromal system of bone and marrow. *Clin Orthop Relat Res* 240, 270-281.

Beresford, J. N., Gallagher, J. A., Poser, J. W., and Russell, R. G. (1984) Production of osteocalcin by human bone cells in vitro. Effects of 1,25(OH)2D3, 24,25(OH)2D3, parathyroid hormone, and glucocorticoids. *Metab Bone Dis Relat Res* 5, 229-234.

Bergmann, G., Deuretzbacher, G., Heller, M., Graichen, F., Rohlmann, A., Strauss, J., and Duda, G. N. (2001) Hip contact forces and gait patterns from routine activities. *J Biomech* 34, 859-871.

Bergmann, G., Siraky, J., and Rohlmann, A. (1984) A comparison of hip joint forces in sheep, dog and man. *J Biomech* 17, 907-921.

Bianco, P. and Robey, P. G. (2000) Marrow stromal cells. *J Clin Invest* 105, 1663-1668.

Birrell, F., Johnell, O., and Silman, A. (1999) Projecting the need for hip replacement over the next three decades: influence of changing demography and threshold for surgery. *Ann Rheum Dis* 58, 569-572.

Björn, S. and Aspenberg, P. (2003) PMMA particles and pressure - a study of the osteolytic properties of two agents proposed to cause prosthetic loosening. *J Orthop Res* 21, 196-201.

Blokhuis, T. J., Wippermann, B. W., den Boer, F. C., van Lingen, A., Patka, P., Bakker, F. C., and Haarman, H. J. T. M. (1999) Resorbable calcium phosphate particles as a carrier material for bone marrow in an ovine segmental defect. *J Biomed Mater Res* 369-375.

Blom, A. W., Cunningham, J. L., Hughes, G., Lawes, T. J., Smith, N., Blunn, G., Learmonth, I. D., and Goodship, A. E. (2005) The compatability of ceramic bone graft substitutes as allograft extenders for use in impaction grafting of the femur. *J Bone Joint Surg* 87-B, 421-425.

Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A., and Nikbin, B. (2006) Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 7, 14.

Bostrom, M., Boskey, A., Kaufman, J. J., and Einhorn, T. A. (2000) Form and Function of Bone. In: **Orthopaedics Basic Science,** pp. 319-369. Eds J. A. Buckwalter, T. A. Einhorn, S. R. Simon.

Bourn, J. (2003) Hip replacements: An update. Report by the comptroller and auditor general. *National Audit Office* HC 956, 1-35.

Boyce, T., Edwards, J., and Scarborough, N. (1999) Allograft bone. The influence of processing on safety and performance. *Orthop Clin North Am* 30, 571-581.

Boyde, A., Corsi, A., Quatro, R., Cancedda, R., and Bianco, P. (1999) Osteoconduction in large macroporous hydroxyapatite ceramic implants: Evidence for a complementary integration and disintegration mechanism. *Bone* 24, 579-589.

Breibart, A. S., Grande, D. A., Kessler, R., Ryaby, J. T., Fitzsimmons, R. J., and Grant, R. T. (1998) Tissue engineered bone repair of calvarial defects using cultured periosteal cells. *Plast Reconstr Surg* 567-574.

Brewster, N. T., Gillespie, W. J., Howie, C. R., Madabhushi, S. P. G., Usmani, A. S., and Fairbairn D.R. (1999) Mechanical considerations in impaction bone grafting. *J Bone Joint Surg Br* 81-B, 118-124.

Bruder, S. P. and Caplan, A. I. (1990a) Osteogenic lineage analysis is facilitated by organ cultures of embryonic chick periosteum. *Dev Biol* 141, 319-329.

Bruder, S. P. and Caplan, A. I. (1990b) Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. *Bone* 11, 189-198.

Bruder, S. P., Fink, D. J., and Caplan, A. I. (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem* 56, 283-294.

Bruder, S. P. and Fox, B. S. (1999) Tissue engineering of bone: cell based strategies. *Clin Orthop Relat Res* 367S, S68-S83.

Bruder, S. P., Jaiswal, N., and Hayensworth, S. E. (1997) Growth Kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during excessive subcultivation and following cryopreservation. *J Cell Biochem* 64, 278-294.

Bruder, S. P. and Caplan, A. I. (1989) First bone formation and the dissection of an osteogenic lineage in the embryonic chick tibia is revealed by monoclonal antibodies against osteoblasts. *Bone* 10, 359-375.

Bruder, S. P., Jaiswal, N., Ricalton, N. S., Mosca, J. D., Kraus, K. H., and Kadiyala, S. (1998a) Mesenchymal stem cells in osteobiology and applied bone regeneration. *Clin Orthop Relat Res* 355S, S247-S256.

Bruder, S. P., Kraus, K. H., Goldberg, V. M., and Kadiyala, S. (1998b) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* 80, 985-996.

Buck, B. E. and Malinin, T. I. (1994) Human bone and tissue allografts: Preparation and safety. *Clin Orthop Relat Res* 303, 8-17.

Buma, P. and Gardeniers, J. W. (1995) Tissue reactions around a hydroxyapatite-coated hip prosthesis. Case report of a retrieved specimen. *J Arthroplasty* 10, 389-395.

Burchardt, H. (1987) Biology of bone transplantation. *Orthop Clin North Am* 18, 187-195.

Cabanela, M. E., Trousdale, R. T., and Berry, D. J. (2003) Impacted cancellous graft plus cement in hip revision. *Clin Orthop Relat Res* 417, 175-182Berry DJ.

Caplan, A. I. (1991) Mesenchymal Stem Cells. *J Orthop Res* 9, -650.

Caplan, A. I. (1994) The mesengenic process. *Clin Plast Surg* 21, 429-435.

Carlisle, E. and Fischgrund, J. S. (2005) Bone morphogenic proteins for spinal fusion. *Spine J* 240S-249S.

Chen, Q. Z., Wong, C. T., Lu, W. W., Cheung, K. M. C., Leong, J. C. Y., and Luk, K. D. K. (2004) Strengthening mechanisms of bone bonding to crystalline hydroxyapatite in vivo. *Biomaterials* 25, 4243-4254.

Chistolini, P., Ruspantini, I., Bianco, P., Corsi, A., Cancedda, R., and Quarto, R. (1999) Biomechanical evaluation of cell-loaded and cell-free hydroxyapatite implants for the reconstruction of segmental bone defects. *J Mater Sci Mater Med* 10, 739-742.

Coathup, M. J., Blackburn, J., Goodship, A. E., Cunningham, J. L., Smith, T., and Blunn, G. W. (2005) Role of hydroxyapatite coating in resisting wear particle migration and osteolysis around acetabular components. *Biomaterials* 26, 4161-4169.

Collignon, H., Davicco, M. J., and Bartlet, J. P. (1997) Isolation of cells from ovine fetal long bone and characterisation of their osteoblastic activities during *in vitro* mineralisation. *Arch Physiol Biochem* 105, 158-166.

Colter, D., Class, R., DiGirolamo, C. M., and Prockop, D. (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *PNAS* 97, 3213-3218.

Conget, P. and Minguell, J. J. (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 181, 67-73.

Cooper, R. A., McAllister, C. M., Borden, L. S., and Bauer, T. W. (1992) Polyethylene debris-induced osteolysis and loosening in uncemented total hip arthroplasty. A cause of late failure. *J Arthroplasty* 7, 285-290.

Daya, S. M., Watson, A., Sharpe, J. R., Giledi, O., Rowe, A., Martin, R., and James, E. (2005) Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. *Opthalmology* 112, 470-477.

Deans, R. J. and Moseley, A. B. (2000) Mesenchymal stem cells: Biology and potential clinical uses. *Exp Hematol* 28, 875-884.

Deleu, J. and Trueta, J. (1965) Vascularisation of bone grafts in the anterior chamber of the eye. *J Bone Joint Surg Br* 47, 319-329.

Devine, S. M., Cobbs, C., Jennings, M., Bartholomew, A., and Hoffman, R. (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into non human primates. *Blood* 101, 2999-3001.

DiMaio, F. R. (2002) The science of bone cement: a historical review. *Orthopedics* 25, 1399-1407.

Dixon, T., Shaw, M., Ebrahim, S., and Dieppe, P. (2003) Trends in hip and knee joint replacement: socioeconomic inequalities and projections of need. *Ann Rheum Dis* 63, 825-830.

Ducy, P., Zhang, R., Geoffroy, A. L., and Karsenty, G. (1997) Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. *Cell* 89, 747-754.

Duda, G. N., Eckert-Hübner, K., Sokiranski, R., Kreutner, A., Miller, R., and Claes, L. (1998) Analysis of inter-fragmentary movements as a function of musculoskeletal loading in sheep. *J Biomech* 31, 201-210.

Einhorn, T. A. (2003) Clinical application of recombinant human BMPs: early experience and future development. *J Bone Joint Surg* 85-A, 82-88.

Eipers, G. P., Kale, S., Taichman, R. S., Pipia, G. G., Swords, N. A., Mann, G. K., and Long, M. W. (2000) Bone marrow accessory cells regulate human bone precursor cell development. *Exp Hematol* 28, 815-825.

Eldridge, J. D., Smith, E. J., Hubble, M. J., Whitehouse, S. L., and Learmonth, I. D. (1997) Massive early subsidence following femoral impaction grafting. *J Arthroplasty* 12, 535-540.

Erices, A., Conget, P., and Minguell, J. J. (2000) Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 109, 235-242.

Ferrari, G., Angelis, G. C.-D., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279, 1528-1530.

Franklin, J., Robertsson, O., Gestsson, J., Lohmander, L. S., and Ingvarsson, T. (2003) Revision and complication rates in 654 Exeter total hip replacements, with a maximum follow up of 20 years. *BMC Musculoskelet Disord* 4, 1471-2474.

Freshney, R. I. (2000) **Culture of animal cells: A manual of basic technique.** John Wiley and Sons Inc.: New York.

Friedenstein, A. J. (1980) Stromal mechanisms of bone marrow: Cloning in vitro and retransplantation in vivo. *Haematology Blood Transfusion* 25, 19-29.

Friedenstein, A. J. (1994) Marrow stromal fibroblasts. *Calcif Tissue Int* 56, S17.

Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 3, 393-403.

Friedenstein, A. J., Gorsaja, J. F., and Kulagina, N. N. (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 4, 267-274.

Friedlaender, G. E., Perry, C. R., Cole, J. D., Cook, S. D., Cierny, G., Muschler, G. F., Zych, G. A., Calhoun, J. H., LaForte, A. J., and Yin, S. (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. *J Bone Joint Surg Am* 83-A Suppl 1, S151-S158.

Giannoudis, P. V., Dinopoulos, H., and Tsiridis, E. (2005) Bone substitutes: an update. *Injury* 36 Suppl 3, S20-S27.

Giannoudis, P. V. and Tzioupis, C. (2005) Clinical applications of BMP-7. The UK perspective. *Injury* 36, S47-S50.

Gie, G. A., Linder, L., Ling, R. S. M., Simon, J. P., Slooff, T. J. J. H., and Timperley, A. J. (1992) Impacted cancellous allografts and cement for revision total hip arthroplasty. *J Bone Joint Surg Br* 75-B, 14-21.

Gie, G. A., Linder, L., Ling, R. S. M., Simon, J. P., Slooff, T. J. J. H., and Timperley, A. J. (1993) Contained morsellised allograft in revision total hip arthroplasty: Surgical technique. *Orthop Clin North Am* 24, 717-725.

Goel, V. K., Drinker, H., Panjabi, M. M., and Strongwater, A. A. (1982) Selection of an animal model for implant fixation studies: anatomical aspects. *Yale J Biol Med* 55, 113-122.

Gokhale, S., Soliman, A., Dantas, J. P., Richardson, J. B., Cook, F., Kuiper, J. H., and Jones, P. (2005) Variables affecting initial stability of impaction grafting for hip revision. *Clin Orthop Relat Res* 174-180.

Goldstein, A. S. (2001) Effect of seeding osteoprogenitor cells as dense clusters on cell growth and differentiation. *Tissue Eng* 7, 817-827.

Gomez, P. F. and Morcuende, J. A. (2005) Early attempts at hip arthroplasty--1700s to 1950s. *Iowa Orthop J* 25, 25-29.

Gosain, A. K., Riordan, P. A., Song, L., Amarante, M. T., Kalantarian, B., Nagy, P. G., Wilson, C. R., Toth, J. M., and McIntyre, B. L. (2003a) A 1-year study of osteoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part II. bioengineering implants to optimize bone replacement in reconstruction of cranial defects. *Plast Reconstr Surg* 114, 1155-1163.

Gosain, A. K., Santoro, T. D., Song, L. S., Capel, C. C., Sudhakar, P. V., and Matloub, H. S. (2003b) Osteogenesis in calvarial defects: contribution of the dura, the pericranium, and the surrounding bone in adult versus infant animals. *Plast Reconstr Surg* 112, 515-527.

Goshima, J., Goldberg, V. M., and Caplan, A. I. (1991) The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. *Clin Orthop Relat Res* 262, 298-311.

Griffon, D. J., Dunlop, D. G., Howie, C. R., Pratt, J. N. J., Gilchrist, T. J., and Smith, N. (2001) An ovine model to evaluate the biologic properties of impacted morselised bone graft substitutes. *J Biomed Mater Res* 56, 444-451.

Gronthos, S., Franklin, D. M., Leddy, H. A., Robey, P. G., Storms, R. W., and Gimble, J. M. (2001) Surface protein characterisation of human adipose tissue-derived stromal cells. *J Cell Physiol* 189, 54-63.

Gronthos, S., Graves, S. E., Ohta, S., and Simmons, P. J. (1994) The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* 84, 4164-4173.

Gundle, R., Joyner, C. J., and Triffit, J. T. (1995) Human bone tissue formation in diffusion chamber culture in vivo by bone-derived cells and marrow stromal fibroblastic cells. *Bone* 16, 597-601.

Gundle, R., Joyner, C. J., and Triffitt, J. T. (1997) Interactions of human osteoprogenitors with porous ceramic following diffusion chamber implantation in a xenogeneic host. *J Mater Sci Mater Med* 8, 519-523.

Hall, P. A. and Watt, F. M. (1989) Stem cells: the generation and maintenance of cellular diversity. *Development* 106, 619-633.

Hancox, N. M. (1972) **Biology of Bone.** Cambridge University Press: Cambridge.

Harris, C. T. and Cooper, L. F. (2004) Comparison of bone graft matrices for human mesenchymal stem cell-directed osteogenesis. *J Biomed Mater Res* 68A, 747-755.

Harris, W. H. (1995) The problem is osteolysis. *Clin Orthop Relat Res* 46-53.

Hayensworth, S. E., Goshima, J., Goldberg, M., and Caplan, A. I. (1992) Characterisation of cells with osteogenic potential from human marrow. *Bone* 13, 81-88.

Heisel, C., Silva, M., and Schmalzried, T. (2003) Bearing surface options for total hip replacement in young patients. *J Bone Joint Surg* 85-A, 1366-1379.

Heliotis, M. and Tsiridis, E. E. (2001) Fresh frozen bone in femoral impaction grafting: can developments in bone regeneration improve on this? *Med Hypotheses* 57, 675-678.

Hernigou, P., Poignard, A., Beaujean, F., and Rouard, H. (2005) Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* 87, 1430-1437.

Holy, C. E., Shoichet, M. S., and Davies, J. E. (2000) Engineering three-dimensional bone tissue *in vitro* using biodegradable scaffolds: Investigating initial cell-seeding density and culture period. *J Biomed Mater Res* 51, 376-382.

Horwitz, E. M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Deans, R. J., Krause, D. S., and Keating, A. (2005) Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 7, 393-395.

Horwitz, E. M., Prockop, D. J., Fitzpatrick, L. A., Koo, W. W., Gordon, P. L., Neel, M., Sussman, M., Orchard, P., Marx, J. C., Pyeritz, R. E., and Brenner, M. K. (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 5, 309-313.

Hughes, F. J. and Aubin, J. E. (1998) Culture of cells of the osteoblastic lineage. In: **Methods in Bone Biology,** pp. 1-49. Eds Arnett T.R., Henderson B. Chaspman & Hall: London.

Hutmacher, D. W. and Sittinger, M. (2003) Periosteal cells in bone tissue engineering. *Tissue Eng* 9, 45-64.

Isacson, J., Stark, A., and Wallensten, R. (2000) The Wagner revision prosthesis consistently restores femoral bone structure. *Int Orthop* 24, 139-142.

Iuchi, S., Dabelsteen, S., Easley, K., Rheinwald, J. G., and Green, H. (2006) Immortalised keratinocyte lines derived from human embryonic stem cells. *PNAS* 103, 1792-1797.

Jacobs, J. J., Goodman, S. B., Sumner, D. R., and Hallab, N. J. (2000) Biologic response to orthopaedic implants. In: **Orthopaedic Basic Science,** pp. 402-426. Eds J. A. Buckwalter, T. A. Einhorn, S. R. Simon.

Jaiswal, N., Hayensworth, S. E., Caplan, A. I., and Bruder, S. P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* 64, 295-312.

Jansen, J., Hanks, S., Thompson, J. M., Dugan, M. J., and Akard, L. P. (2005) Transplantation of hematopoietic stem cells from the peripheral blood. *J Cell Mol Med* 9, 37-50.

Jessop, H. L., Nobel, B. S., and Cryer, A. (1994) The differentiation of a potential mesenchymal stem cell population within ovine bone marrow. *Biochem Soc Trans* 22, 248.

Kadiyala, S. and Jaiswal, N. (1997) Culture-expanded, bone marrow-derived mesenchymal stem cells can regenerate a critical-sized segmental bone defect. *Tissue Eng* 3, 173-185.

Kang, K. S., Kim, S. W., Oh, Y. H., Yu, J. W., Kim, K. Y., Park, H. K., Song, C. H., and Han, H. (2005) A 37-year-old spinal cord injured patient, transplanted of multipotent stem cells from human UC blood, with improved sensory perception

and mobility, both functionally and morphologically: a case study. *Cytotherapy* 7, 368-373.

Karsenty, G., Ducy, P., Starbuck, M., Priemel, M., Shen, J., Geoffroy, V., and Amling, M. (1999) Cbfa1 as a regulator of osteoblast differentiation and function. *Bone* 25, 107-108.

Kenwright, J. and Goodship, A. E. (1989) Controlled mechanical stimulation in the treatment of tibial fractures. *Clin Orthop Relat Res* 241, 36-47.

Khan, S. N., Cammisa, F. P., Sandhu, H. S., Diwan, A. D., Girardi, F. P., and Lane, J. M. (2005) The biology of bone grafting. *J Am Acad Orthop Surg* 13, 77-86.

Khosla, S. (2001) Minireview: The OPG/RANKL/RANK system. *Endocrinology* 142, 5050-5055.

Kon, E., Muraglia, A., Corsi, A., Bianco, P., Marcacci, M., Martin, I., Boyde, A., Ruspantini, I., Chistolini, P., Rocca, M., Giardino, R., Cancedda, R., and Quarto, R. (2000) Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res* 49, 328-337.

Korda, M., Blunn, G. W., Phipps, K., Rust, P., Di-Silvio, L., and Coathup, M. J. (2006) Can Mesenchymal Stem Cells Survive under Normal Impaction Forces in Revision Total Hip Replacements. *Tissue Eng* 12, 625-630.

Krishnanmurthy, A. B., MacDonald, S. J., and Paprosky, W. G. (1997) 5 to 13 year follow up study on cementless femoral components in revision surgery. *J Arthroplasty* 12, 839-837.

Kruyt, M. C., de Bruijn, J. D., Wilson, M. S., Oner, F. C., van Blitterswijk, C. A., Verbout, A. J., and Dhert, W. J. A. (2003) Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in goats. *Tissue Eng* 9, 327-335.

Kruyt, M. C., de Bruijn, J. D., Yuan, H., van Blitterswijk, C. A., Verbout, A. J., Oner, F. C., and Dhert, W. J. (2004a) Optimization of bone tissue engineering in goats: a peroperative seeding method using cryopreserved cells and localized bone formation in calcium phosphate scaffolds. *Transplantation* 77, 359-365.

Kruyt, M. C., Dhert, W. J. A., Oner, C., van Blitterswijk, C. A., and Verbout, A. J. (2004b) Optimization of bone-tissue engineering in goats. *J Biomed Mater Res* Applied Biomaterials 69B, 113-120.

Kruyt, M. C., Dhert, W. J. A., Yuan, H., Wilson, C. R., van Blitterswijk, C. A., Verbout, A. J., and de Bruijn, J. D. (2004c) Bone tissue engineering in critical size defect compared to ectopic implantations in the goat. *J Orthop Res* 22, 544-551.

Kuznetsov, S. A., Mankini, M. H., Gronthos, S., Satomura, K., Bianco, P., and Robey, P. G. (2001) Circulating skeletal stem cells. *J Cell Biol* 153, 1133-1139.

Lackshmipathy, U. and Verfaillie, C. (2005) Stem cell plasticity. *Blood Rev* 19, 29-38.

Lacroix, D. and Prendergrast, P. J. (2002) A mechano-regulation for tissue differentiation during fracture healing: analysis of gap size and loading. *J Biomech* 35, 1163-1171.

Lane, J. M. (2001) BMPs: Why are they not in everyday use? *J Bone Joint Surg Am* 83, 161-162.

Lane, J. M. and Sandhu, H. S. (1987) Current approaches to experimental bone grafting. *Orthop Clin North Am* 18, 213-225.

Lanza, R. P., Langer, R., and Vacanti, J. P. (2000) **Principles of tissue engineering.** Academic Press: London.

Lennon, D. P., Hayensworth, S. E., Arm, D. M., Baber, M. A., and Caplan, A. I. (2000) Dilution of human mesenchymal stem cells with dermal fibroblasts and the effects on in vitro and in vivo osteochondrogenesis. *Dev Dyn* 219, 50-62.

Leopold, S. S., Jacobs, J. J., and Rosenburg, A. G. (2000) Cancellous allograft in revision total hip arthroplasty: A clinical review. *Clin Orthop Relat Res* 86-97.

Leopold, S. S., Rosenburg, A. G., Bhatt, R. D., Sheinkop, M. B., Quigley, L. R., and Galante, J. O. (1999) Cementless acetabular revision. Evaluation at an average of 10.5 years. *Clin Orthop Relat Res* 369, 179-186.

Linder, L. (2000) Cancellous impaction grafting in the human femur. *Acta Orthop Scand* 71, 543-552.

Ling, R. S. (1997) Femoral component revision using impacted morsellised cancellous graft. *J Bone Joint Surg Br* 79, 874-875.

Ling, R. S., Timperley, A. J., and Linder, L. (1993) Histology of cancellous impaction grafting in the femur. A case report. *J Bone Joint Surg Br* 75, 693-696.

Logeart-Avramoglou, D., Anagnostou, F., Bizios, R., and Petite, H. (2004) Engineering bone: challenges and obstacles. *J Cell Mol Med* 9, 72-84.

Martini, L., Fini, M., Giavaresi, G., and Giardino, R. (2004) Sheep model in orthopaedic research: A literature review. *Comp Med* 51, 292-299.

Masterson, E. L., Masri, B. A., and Duncan, C. P. (1997) The cement mantle in the Exeter impaction allografting technique: A cause for concern. *J Arthroplasty* 12, 759-764.

McGee, M. A., Findlay, D. M., Howie, D. W., Carbone, A., Ward, P., Stamenkov, R., Page, T. T., Bruce, W. J., Wildenauer, C. I., and Carol, T. (2004) The use of OP-1 in femoral impaction grafting in a sheep model. *J Orthop Res* 22, 1008-1015.

McPherson, J. M. and Tubo, R. (2000) Articular Cartilage Injury. In: **Principles of Tissue Engineering,** pp. 697-709. Eds R. P. Lanza, R. Langer, J. Vacanti. Academic Press: London.

Meding, J. B., Ritter, M. A., Keating, E. M., and Faris, P. M. (1997) Impaction bone-grafting before insertion of a femoral stem with cement in revision total hip arthroplasty. A minimum two-year follow-up study. *J Bone Joint Surg Am* 79, 1834-1841.

Mikhail, W. E., Weidenhielm, L. R., Wretenberg, P., Mikhail, N., and Bauer, T. W. (1999) Femoral bone regeneration subsequent to impaction grafting during hip revision: histologic analysis of a human biopsy specimen. *J Arthroplasty* 14, 849-853.

Mikuni-Takagaki, Y., Suzuki, Y., Kawase, T., and Saito, S. (1996) Distinct responses of different populations of bone cells to mechanical stress. *Endocrinology* 137, 2028-2035.

Minguell, J. J., Erices, A., and Conget, P. (2001) Mesenchymal stem cells. *Exp Biol Med* 226, 507-520.

Muraglia, A., Cancedda, R., and Quarto, R. (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* 113, 1161-1166.

Muschler, G. F. and Midura, R. J. (2002) Connective tissue progenitors: practical concepts for clinical appllications. *Clin Orthop Relat Res* 395, 66-80.

Muschler, G. F., Nitto, H., Boehm, C. A., and Easley, K. A. (2001) Age and gender related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. *J Orthop Res* 19, 117-125.

Mushipe, M. T., Chen, X., Jennings, D., and Li, G. (2006) Cells seeded on MGB scaffold survive impaction grafting technique: potential application of cell-seeded biomaterials for revision arthroplasty. *J Orthop Res* 24, 501-507.

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108, 17-29.

Nelissen, R. G., Bauer, T. W., Weidenhielm, L. R. A., LeGolvan, D. P., and Mikhail, M. (1995) Revision hip arthroplasty with the use of cement and impaction grafting. Histological analysis of four cases. *J Bone Joint Surg Am* 77, 412-422.

Newman, P. H. (1971) Development of total hip replacement. In: **Total Hip Replacement,** pp. 13-25. Ed M. Jayson. Sector Publishing Ltd: London.

Niklason, L. E. (2000) Engineering of bone grafts. *Nat Biotechnol* 18, 929-930.

Norman-Taylor, F. H., Santori, N., and Villar, R. N. (1997) The trouble with bone allograft. *Br Med J* 315, 498.

Ohgushi, H., Goldberg, V. M., and Caplan, A. I. (1989) Repair of bone defects with marrow cells and porous ceramic. Experiments in rats. *Acta Orthop Scand* 60, 334-339.

Okumura, M., Ohugushi, H., Dohi, Y., Katuda, T., Tamai, S., Koerten, H. K., and Tabata, S. (1996) Osteoblastic phenotype expression on the surface of hydroxyapatite ceramics. *J Biomed Mater Res* 37, 122-129.

Oonishi, H. (1991) Orthopaedic applications of hydroxyapatite. *Biomaterials* 12, 171-178.

Oonishi, H., Kadoya, Y., Iwaki, H., and Kin, N. (2000) Hydroxyapatite granules interposed at bone-cement interface in total hip replacements: Histological study of retrieved specimens. *J Biomed Mater Res* 53, 174-180.

Organogenesis, I. (2006) Frequently asked questions about apligraf®. *http://www apligraf com/index.*

Orkin, S. H. and Morrison, S. J. (2002) Stem-cell competition. *Nature* 418, 25-27.

Owen, M. (1985) Lineage of osteogenic cells and their relationship to the stromal system. *J Bone Miner Res* 3, 1-25.

Owen, M. and Friedenstein, A. J. (1988) Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 136, 42-60.

Park, S.-H., Llinás, A., Goel, V. K., and Keller, J. C. (1995) Hard Tissue Replacements. In: **The Biomedical Engineering Handbook,** pp. 672-703. Ed J. D. Bronzino. CRC Press: Hartford.

Park, S. E., Oreffo, R. O. C., and Triffitt, J. T. (1999) Interconversion potential of cloned human marrow adipocytes in vitro. *Bone* 24, 549-554.

Pastoureau, P. M. E., Arlot, F., Caulin, J. P., Barier, J. P., Meunier, P. J., and Delmas, P. D. (1989) Effects of oophorectomy on biochemical and histological indices of bone turnover in ewes. *J Bone Miner Res* 4, 237.

Patel, A. N., Geffner, L., Vina, R. F., Saslavsky, J., Urschel, H. C., Kormos, R., and Benetti, F. (2005) Surgical treatment for congestive heart failure with autologous adult stem cell transplantation: A prospective randomized study. *J Thorac Cardiovasc Surg* 130, 1631-1638.

Pelker, R. R. and Friedlaender, G. E. (1987) Biomechanical aspects of bone autografts and allografts. *Orthop Clin North Am* 18, 235-239.

Pelker, R. R., Friedlander, G. E., and Markham, T. C. (1983) Biomechanical properties of bone allografts. *Clin Orthop Relat Res* 174, 54-57.

Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C., Bourguignon, M., Oudina, K., Sedel, L., and Guillemin, G. (2000) Tissue-engineered bone regeneration. *Nat Biotechnol* 18, 959-963.

Phinney, D. J., Kopen, G., Righter, W., Webster, S., Tremain, N., and Prockop, D. (1999) Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem* 75, 424-436.

Phipps, K. (2004) The engineering and biology of femoral impaction grafting. *Thesis* 1-219.

Phipps, K., Saksena, J., Muirhead-Allwood, S., Miles, A. W., Goodship, A., and Blunn, G. W. (2002) Intra-operative force measurement of impaction allografting. *EORS 12th Annual Meeting, Lausanne* 0-1.

Pietrzak, W. S., Woodell-May, J., and McDonald, N. (2006) Assay of bone morphogenic protein-2, -4, and -7 in human demineralised bone matrix. *J Craniofac Surg* 17, 84-90.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas R.Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143-146.

Price, M. J., Chou, C.-C., Frantzen, M., Miyamoto, T., Kar, S., Lee, S., Shah, P. K., Martin, B. J., Lill, M., Forrester, J. S., Chen, P.-S., and Makkar, R. R. (2005) Intravenous mesenchymal stem cell therapy early after reperfused acute myocardial infarction improves left ventricular function and alters electrophysiologic properties. *Inter J Cardiol* 111, 231-239.

Prockop, D. J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276, 71-74.

Reis, R. S., Neves, I., Jr., Lourenco, S. L., Fonseca, L. S., and Lourenco, M. C. (2004) Comparison of flow cytometric and Alamar Blue tests with the proportional method for testing susceptibility of Mycobacterium tuberculosis to rifampin and isoniazid. *J Clin Microbiol* 42, 2247-2248.

Rickard, D. J., Kassem, M., Hefferan, T. E., Sarkar, G., Spelsberg, T. C., and Riggs, L. (1996) Isolation and characterisation of osteoblast precursor cells from human bone marrow. *J Bone Miner Res* 11, 312-323.

Ripamonti, U. (1991) The induction of bone in osteogenic composites of bone matrix and porous hydroxyapatite replicas: an experimental study on the baboon (Papio ursinus). *Journal of Oral and Maxillofacial Surgery* 49, 817-830.

Sabokbar, A., Fujikawa, Y., Neale, S., Murray, D. W., and Athanasou, N. A. (1997) Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. *Ann Rheum Dis* 56, 414-420.

Salgado, A. J., Coutinho, O. P., and Reis, R. L. (2004) Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 4, 743-765.

Scaglione, S., Braccini, A., Wendt, D., Jaquiery, C., Beltrame, F., Quarto, R., and Martin, I. (2005) Engineering of osteoinductive grafts by isolation and expansion of ovine bone marrow stromal cells directly on 3D ceramic scaffolds. *Biotechnol and Bioeng* 93, 181-187.

Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. m., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R., and Gearhart, J. D. (1998) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci U S A* 95, 13726-13731.

Shamblott, M. J., Edwards, B. E., and Gearhart, J. D. (2000) Pluripotent stem cells. In: **Principles of tissue engineering,** pp. 369-381. Eds R. P. Lanza, R. Langer, J. Vacanti. Academic Press: London.

Shanbhag, A. S., Jacobs, J. J., Glant, T. T., Gilbert, J. L., Black, J., and Galante, J. O. (1993) Composition and morphology of wear debris in failed uncemented total hip replacement. *J Bone Joint Surg Br* 76-B, 60-67.

Simmons, P. J. and Torok-Strob, B. (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody. *Blood* 78, 55-62.

Sloof, T. J. J. H., Huiskes, R., van Horn, J., and Lemmens, A. J. (1984) Bone grafting in total hip replacement for acetabular protrusion. *Acta Orthop Scand* 55, 593-596.

Soballe, K., Hansen, E. S., Rasmussen, H., Jorgensen, P. H., and Bunger, C. (1992) Tissue ingrowth into titanium and hydroxyapatite-coated implants during stable and unstable mechanical conditions. *J Orthop Res* 10, 285-299.

Solheim, E. (1998) Growth factors in bone. *Int Orthop* 22, 410-416.

Sporer, S. M. and Paprosky, W. G. (2004) Femoral fixation in the face of considerable bone loss. The use of modular stems. *Clin Orthop Relat Res* 429, 227-231.

Stephenson, P. K., Freeman, M. A. R., Revell, P. A., Germain, J., Tuke, M., and Pirie, C. J. (2003) The effect of hydroxyapatite coating on ingrowth into cavities in an implant. *J Arthroplasty* 6, 51-58.

Tachibana, Y., Ninomiya, S., Kim, Y. T., and Sekikawa, M. (2003) Tissue response to porous hydroxyapatite ceramic in the human femoral head. *J Orthop Sci* 8, 549-553.

Tägil, M. (2000) The morselized and impacted bone graft. Animal experiments on proteins, impaction and load. *Acta Orthop Scand Suppl* 290, 1-40.

Tägil, M. and Aspenberg, P. (1998) Impaction of cancellous bone grafts impairs osteoconduction in titanium chambers. *Clin Orthop Relat Res* 232, 231-238.

Tomford, W. W., Mankin, H. J., Friedlaender, G. E., Doppelt, S. H., and Gebhardt, M. C. (1987) Methods of banking bone and cartilage for allograft transplantation. *Orthop Clin North Am* 18, 241-247.

Torricelli, P., Fini, M., Giavaresi, G., Rocca, M., Pierini, G., and Giardino, R. (2000) Isolation and characterization of osteoblast cultures from normal and osteopenic sheep for biomaterials evaluation. *J Biomed Mater Res* 52, 177-182.

Ullmark, G. and Obrant, K. J. (2002) Histology of impacted bone-graft incorporation. *J Arthroplasty* 17, 150-157.

Urist, M. R. (2002) Bone:Formation by autoinduction. *Clin Orthop Relat Res* 395, 4-10.

Vacanti, C. (2004) Cells for building: In tissue engineering, embryonic stem cells might not be the way to go. *The Scientist* 18, 22.

van der Meulin, J., Lewsey, J., Carpendale, C., Davies, F., Pickford, M., Carter, D., Hasler, S., Calcutt, I., Pegg, D., Hoare, A., Newell, C., Fisher, P., Gregg, P., Porter, M., Wilton, T., Thomson, C., Crosbie, A., Borroff, M., Timperley, J., and Howard, P. (2004) National Joint Registry for England and Wales 1st annual report. *National Joint Registry for England and Wales* 1st, 1-102.

van Eeden, S. P. and Ripamonti, U. (1993) Bone differentiation in porous hydroxyapatite in baboons is regulated by the geometry of the substratum: Implications for reconstructive craniofacial surgery. *Plast Reconstr Surg* 93, 959-966.

Van Gaalen, S., Dhert, W. J. A., Kruyt, M. C., Terlou, M., Yuan, H., Oner, F. C., van Blitterswijk, C. A., Verbout, A. J., and de Bruijn, J. D. (2004) Goat bone tissue engineering: The effect of implant location. *EORS 14th Annual Meeting* 14, O-90.

Wan, C., He, Q., McCaigue, M., Marsh, D., and Li, G. (2006) Nonadherent cell population of human marrow culture is a complementary source of mesenchymal stem cells (MSCs). *J Orthop Res* 24, 21-28.

Wang, J.-S., Tägil, M., and Aspenberg, P. (1999) Load-bearing increases new bone formation in impacted and morselised allografts. *Clin Orthop Relat Res* 378, 274-281.

Warejcka, D. J., Harvey, R., Taylor, B. J., Young, H. E., and Lucas, P. A. (1996) A population of cells isolated from rat heart capable of differentiating into several mesodermal phenotypes. *J Surg Res* 62, 233-242.

Watt, F. M. and Hogan, B. L. M. (2000) Out of Eden: Stem cells and their niches. *Science* 287, 1427-1430.

Weyts, F. F. A., Bosmans, B., Niesing, R., van Leeuwen, J. M. P. T., and Weinans, H. (2003) Mechanical control of human osteoblast apoptosis and proliferation in relation to differentiation. *Calcif Tissue Int* 72, 505-512.

Wright, T. M. and Li, S. L. (2000) Biomaterials. In: **Orthopaedics Basic Science,** pp. 181-215. Eds J. A. Buckwalter, T. A. Einhorn, S. R. Simon.

Wülling, M., Delling, G., and Kaiser, E. (2003) The origin of the neoplastic stromal cell in giant cell tumour of bone. *Hum Pathol* 34, 983-993.

Yamaski, H. and Sakai, H. (1991) Osteogenic responses to hydroxyapatite ceramics under the skin of dogs. *Biomaterials* 13, 308-312.

Yang, X., Tare, R. S., Partridge, K. A., Roach, H. I., Carke, N. M. P., Howdle, S., Shakesheff, K. M., and Oreffo, R. O. C. (2003) Induction of human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation by osteoblast stimulating factor-1/Pleiotrophin: Osteoconductive biomimetic scaffolds for tissue engineering. *J Bone Joint Surg* 18, 47-57.

Yoshikawa, T., Ohgushi, H., and Tamai, S. (1996) Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. *J Biomed Mater Res* 32, 481-492.

Young, B. W. and Heath, J. W. (2000) **Wheater's Functional Histology: a text and colour atlas.** Churchill Livingston: London.