Augmenting Osseointegration Of Implants Using Bone Marrow Stromal Cells

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I, Sujith Konan confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Introduction

The greatest challenge facing the success of orthopaedic implants is improving their fixation to bone to enhance their longevity. Bone marrow stromal cells (BMSC), are a population of plastic-adherent cells derived from the bone marrow. The main hypothesis of this thesis is that viable BMSC can be applied to implants using a fibrin glue-spray system; and increase bone formation adjacent to the implants and improve bone-implant contact.

Methods

The experiments were undertaken in a large animal model. Four scenarios were tested

1) The ability of BMSC to improve implant fixation using models of total hip replacement, massive endoprosthetic replacement and bone defect around pins.

2) The effect of varying cell dosages of BMSC in their ability to produce new bone and improve bone implant contact.

3) The effect of differentiating the BMSC along the osteogenic pathway in their ability to produce new bone and improve bone implant contact.

4) The effect of using semi-permeable barriers around BMSC sprayed on implants to prevent cell migration

Results

1) BMSC sprayed on the surface of implants resulted in increased bone formation in the total hip replacement, massive endoprosthetic replacement and bone defect around pin models.
2) Bone formation was higher with osteogenic $10x10^6$ BMSC (112.67 ± 30.75

mm^2) compared to osteogenic $2x10^6$ BMSC (76.84 ± 2.25 mm^2). No significant difference was noted in bone formation between undifferentiated $1x10^5$ BMSC (30.76 ± 9.43%) and undifferentiated $10x10^6$ BMSC (28.27 ± 14.64%).

3) Osteogenic differentiated $10x10^6$ BMSC (112.67 ± 30.75 mm^2) produced more bone than undifferentiated $10x10^6$ BMSC (58.22 ± 17.22 mm^2).
4) Using semipermeable barriers resulted in significantly increased bone formation when undifferentiated $1x10^5$ BMSC (61.32 ± 6.94% vs 30.76 ± 9.43%) or undifferentiated $10x10^6$ BMSC (57.46 ± 4.39% vs 28.27 ± 14.64%) was used. This difference was not noted when osteogenic differentiated $10x10^6$ BMSC was used.

The experiments confirm that viable BMSC can be successfully isolated from bone marrow aspiration, differentiated along the osteogenic pathway and sprayed on the surface of various orthopaedic implants to improve bone-implant contact.

Conclusion

This technique of using BMSC may be an ideal alternative to improve osseointegration of implants in challenging clinical scenarios with deficient bone stock.

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Candidates Contributions

I hereby declare that I was involved with the work described in this thesis including cell aspiration and culture, surgical procedures, histology, microscopic analysis and data analysis. I have been guided and helped in my work by my supervisors Professor Blunn and Dr Melanie Coathup as well as Dr Priya Kalia.

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Chapter 1

1. Introduction

The use of implants has revolutionised orthopaedic surgery. One of the greatest challenges facing the success of orthopaedic implants is improving their fixation to bone to enhance longevity. Other challenges include; selection of the most appropriate bearing surface; restoring biomechanics by accurate implant positioning and dealing with inadequate bone stock in revision surgery or following resection of bone for tumor. The following chapters describe the use of bone marrow stromal cells (BMSC) suspended in fibrin glue and sprayed on the surface of implants to enhance their fixation. The experiments were undertaken in a large animal model. The use of autologous BMSC suspended in fibrin glue to produce new bone and enhance implant fixation was investigated first. Following this the effects of various concentrations of BMSC and their interaction with a semi-permeable membrane [polytertrafluroethylene (PTFE)] used for guided bone regeneration was studied. Finally, the role of BMSC differentiated in an osteogenic supplemented media was studied.

The main hypothesis of this thesis is that the longevity of orthopaedic metal implants can be increased by intra-operatively coating their surface with autologous BMSC-fibrin suspension.

This introduction provides an overview of the use of prosthetic replacements in various challenging clinical situations and their modes of failure. The experimental and clinical use of bone marrow stromal cells (BMSC) is reviewed as well as the use of alternative sources to BMSC.

1.1 Total hip replacements

The biggest landmark in total hip replacement (THR) came with the development in the

1960s of the low friction arthroplasty concept by Sir John Charnley (Charnley, 1972). After first using a polytetrafluoroethylene (PTFE) cup, 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. Although Charnely used acrylic bone cement to fix his implants to the bone, currently both cemented and cementless fixation options of total hip replacements are available. The combinations of bearing surfaces available 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 and ceramic on polyethelene combinations of head and liner (Heisel et al., 2004).

1.2 Challenges facing the future of THRs

Total hip replacement (THR) is regarded as one of the most successful advances in modern orthopaedic surgery, with over a 90% clinical success rate at 10 years (Bellamy et al., 1988). In the UK, 50,000 total hip replacements (1 million worldwide) are carried out each year at a cost to the NHS of more than £140 million per annum, an increase of 18% since 1991 (Report by the controller and Auditor General, 2000). This number is set to increase by up to 50% by 2026 due to demographic changes in the population (Birrell et al., 1999). Forty percent of surgeons are now willing to carry out this procedure on young and active patients; which contributes to the growing requirement for total hip replacement surgery (Dixon et al., 2004). Younger patients

with high expectations not only live longer but place high demands on their joint replacements. The increasing number of total hip replacements, especially in the young, has led to a 100% increase in revision surgery due to implant failure since 1991. In the UK, approx. 9,000 (18%) of all hip surgery procedures are now revisions (Dixon et al., 2004). The American Academy of Orthopaedic Surgeons (AAOS) has estimated that the number of revision total hip arthroplasties will continue to rise at a rate of 20% to 30% per year over the next three decades (Frankowski, 2002). The average unit cost of revision surgery according to the Health and Social Care Information Centre (www.doh.gov.uk) is £6,568. Thus on average, the NHS spends £59.1 million on costs associated with revising loose hip components.

Revision hip procedures cost more to perform than primary procedures (average unit cost =£4,744.00). Recent studies have reported that the mean operative time, the estimated blood loss, the complication rate and mean hospital stay are all longer for patients undergoing revision surgery when compared with primary hip surgery (Bozic et al.), (Crowe et al., 2003). In comparison with primary procedures the success of revision hip replacements are reduced. Approximately 25% - 50% of revision procedures will fail and patients with extensive bone loss often face multiple problematic and unsuccessful revision hip replacements as a result of inadequate femoral and acetabular component fixation (Sporer and Paprosky, 2005), (Krishnamurthy et al., 1997). The survivorship of these prostheses is reduced with each operation and bone loss is further exacerbated (Malchau et al., 1993), (Slooff et al., 1998). Thus the annual NHS expenditure is increased. Stable fixation and successful clinical outcomes are dependent on bony support for the implant. Regeneration of bone adjacent to the implants would reduce component failure. Aseptic loosening of the

acetabular component is the major cause of failure in total hip replacement surgery (Berry et al., 2002), (Wroblewski and Siney, 1993).

1.3 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 alpha (TNF-a), 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 trans-membrane receptor, Receptor Activator of Nuclear factor kappa B ligand (RANKL), is expressed on the surface of pre-osteoblastic 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 influenced more by biomechanical stimuli than the cement particles (Skoglund 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. 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.

1.4 Massive Endoprosthetic Replacements

Another common clinical scenario where achieving bone implant osseointegration is crucial is when using endoprosthetic replacements following bone resection for tumor.

A combination of factors such as early detection of tumours, the increased use of chemotherapy, radiotherapy, and improved surgical techniques has resulted in increased

survival of bone tumor patients (Capanna et al., 1994); (Cannon, 1997); (Grimer et al., 1999); (Horowitz et al., 1993). Adjuvant chemotherapy (given post-operatively) has reduced the probability of recurrence and/or metastases (Eckardt et al., 1991b); (Uchida et al., 1997). The surgical options available for the management of the segmental defect created during tumour excision, in order to preserve the limb are: arthrodesis with auto/allograft; vascularized fibular autograft, resection and reinsertion of bone after sterilization; and arthroplasty using either auto/allograft, synthetic graft or massive endoprostheses, including extendible prostheses for growing patients (Schindler et al., 1997); (Unwin et al., 1996).

Routine use of bone graft has its limitations in everyday clinical practice. It is frequently difficult to obtain sufficient amounts of autograft for bone tumour resection sites. Donor site morbidity can be an issue (Abudu et al., 1996). Allografts suffer similar problems in terms of supply, disease transfer from donor to patient, graft failure and fracture, as well as issues with non-union of the graft to the host bone (Gebhardt et al., 1991).

The options excluding the use of bone grafts following large segment bone resections are amputation or endoprosthetic replacements. The latter have the obvious advantage of preserving the function and aesthetics of the original limb, while allowing immediate post-operative weight bearing (Grimer et al., 1999). Studies have attempted to determine the long-term effects of amputation and endoprosthetic replacement on tumour recurrence, functionality of the limb post-replacement and patient satisfaction. Results showed that limb-salvage procedures result in the same rate of disease-free survival as amputations after 10 years, with no significant difference in patient satisfaction in most cases (Rougraff et al., 1994); (Sugarbaker et al., 1982); (Weddington et al., 1985). Some studies state a patient preference for limb salvage over amputation (Harris et al., 1990), and a study by (Grimer et al., 1999) indicated that endodoprosthetic reconstructions are more cost effective than amputation.

The massive implants for limb-salvage surgery are either custom-made or modular (Cannon, 1997) and generally use stems that are cemented into the intramedullary canals with polymethylmethacrylate (PMMA). Uncemented stems are also used (Blunn et al., 2000); (Capanna et al., 1994). An alternative to the use of intramedullary stems is triplate fixation, where extracortical fixation can be used if there has been a large resection, and an insufficient amount of bone remains in which to cement an implant (Coathup et al., 2000); (Cobb et al., 2005). Femoral or tibial endoprostheses may also include a knee joint replacement when the bone tumour has spread to the knee or if there is possible contamination of the joint or synovium, and en bloc resection of the joint is required (Sim and Chao, 1979); (Walker et al., 1981).

1.4.1 Failure of massive endoprosthetic replacements

When compared with the success of joint replacements massive implants are not as successful (Blunn et al., 2000). The Swedish Hip registry found that the survival rate of total hip arthroplasties (THAs) over a ten-year period is 92% (Malchau et al., 2005). By comparison, a ten-year follow up study of massive implants with cemented intramedullary stems found that 93.8% of proximal femoral, 67.4% of distal femoral and 58% of proximal tibial prostheses survived ten years (Unwin et al., 1996). (Scales et al., 1984) investigated the causes of failure for Stanmore cemented massive implants in the 1980's and found that 5.4% of all endoprosthetic reconstructions resulted in infection, 2.7% resulted in implant fracture, and 1.9% failed due to aseptic loosening. Infection was reported to be more common in primary massive endoprosthetic replacements than with conventional joint replacements (Malawer and Chou, 1995). (Capanna et al., 1994) found that the infection rate of distal femoral replacements, using the Kotz modular femoral and tibial resection system (KMFTR) was highest when most

or all of the rectus and vasti muscles had been excised (Capanna et al., 1994). Infection has been a major concern for proximal tibial replacements. Bradish et al. (1987) reported a 7.5% infection rate in distal femoral replacements; whereas Roberts et al., (1991) found that, in 135 distal femoral replacements, 6.8% of all implants suffered from infection, whereas 6% failed due to aseptic loosening. A study by the Royal Orthopaedic Hospital in Birmingham found that up to 13.8% of proximal tibial reconstructions performed between 1977 and 1988 became infected in the first five years (Grimer et al., 1999). In 1995, (Malawer and Chou, 1995) reported a high rate of infection with proximal tibial replacements. Modern surgical techniques have reduced the overall infection rate suffered previously with this type of procedure. For example, in proximal tibial replacements, retention or replacement of lost musculature around the knee by use of a medial gastrocnemius flap has reduced the above-mentioned infection rate from 33% to 12% (Grimer et al., 1999). Improved surgical techniques that reduce incidences of infection and improved implant design have reduced the rate of implant survival, so that aseptic loosening is now considered the major cause of failure of cemented massive implants (Ward et al., 1997). Although mechanical complications are the most common cause of implant failure, infection is the leading cause of both complication and amputation (Shehadeh et al., 2010). The risk of infection increases substantially with revision surgery. As patients may be immunocompromised and surgical procedures tend to be complex and prolonged, infection rates are higher than routine implant surgery.

As a result of adjuvant chemotherapy, there has been an increase in the number of young limb salvage patients. This has affected the results of more recent studies, as the methods that were developed for salvaging low-grade lesions in older patients are now routinely used in younger more active patients with wider resections for larger, more aggressive tumours. A study looking at distal femoral replacements, found that between

1959-1980, the survival rate of proximal femoral replacements was 83 ± 8%, whereas from 1980-1991, the survival rate was reduced to 64 ± 27% (Cobb, 1991).

A study (Unwin et al., 1996) looking at the outcome of 1001 patients with endoprosthetic replacement, mostly as a result of osteosarcoma, osteoclastoma, and chondrosarcoma, showed that over a ten-year period, although 93.8% of proximal femoral replacements survived a ten-year period, only 67.4% of distal femoral replacements and 54.8% of proximal tibial implants survived. Seventy-one of the 1001 patients (7%) required implant revisions for component loosening. The study also found that patients under 20 years of age with more than 60% of bone resected from the distal femur or proximal tibia had the worst prognosis for implant survival. The amount of bone resected is also associated with failure in proximal tibial replacements (Unwin et al., 1996). The long-term study of implant loosening by (Unwin et al., 1996) looking at 493 distal femoral, 263 proximal femoral and 245 proximal tibial replacements, found a high incidence of aseptic loosening.

Failure of the humeral prosthesis due to aseptic loosening is rare and is probably due to a lack of heavy weight bearing in that limb (Cannon, 1997). Uncemented implants, using the Kotz system, did not appear to show similar levels of aseptic loosening, although there were some cases of implant fracture and infection (Capanna et al., 1994). (Horowitz et al., 1993) reported a 25% failure of distal femoral and proximal tibial implants within the first five-years post-operatively in sarcoma patients. (Unwin et al., 1996) comparing survival data on 668 Stanmore femoral replacements (221 proximal and 447 distal femoral implants), found that the success of proximal femoral replacements over ten years was 92%, whereas the survival of distal femoral replacements was 72% when loosening as the only mode of failure was considered.

Loosening in this study was defined as when an implant became unstable in the femur, whether it was due to infection, mechanical forces, or bone remodelling in any replacement that required revision or resulted in amputation of the limb (Unwin et al., 1996).

Diaphyseal femoral and tibial implants also have similar loosening rates. Abudu et al., (1996) reported approximately one-third of their diaphyseal implants failing due to symptomatic mechanical loosening. One mode of failure is thought to result from torsional forces caused by the use of a fixed hinge knee component in certain massive implants (Roberts et al., 1991); (Unwin et al., 1996). The use of a rotating hinge has reduced the rate of aseptic loosening (Unwin et al., 1996). However, this is not the only cause of aseptic loosening responsible for implant failure.

Studies by Blunn et al. (2000) and Unwin et al. (1996) have detailed a possible mechanism for aseptic loosening. In the case of massive implants, an offset exists between the implant stem and the axis of the femur from the femoral head to the centre of the knee. This offset is thought to cause a bending moment within the cemented fixation. Due to the line of load this offset is more pronounced proximally on the femur than distally and, thus, stems that are cemented more proximally in the bone suffer from greater bending forces within the cement mantle.

As distal femoral replacements have more proximal transection sites and subsequent cement fixation than proximal femoral implants, they have a greater offset to the axis of the longbone. This is also the case with lengthier tumour resection, where the upper stem may be placed more proximally in the bone. The increased force from the bending caused by this offset eventually causes a deterioration of the bone-cement interface, which slowly progresses from the transection site to the stem tip of the implant (Unwin et al., 1996).

Loosening begins with osteolysis adjacent to the implant shoulder (Blunn and Wait, 1991), (Ward et al., 1997), which can occur within six months post-operatively. Over time, osteolysis is seen to progress along the bone-cement interface, and radiographically can be viewed as radolucent lines.

Cobb (Cobb, 1991) reported a deterioration of the interface between bone and cement, which correlated with decreased function of the implant. There was a steady deterioration of the interface and there was only a 56% probability of a good interface eight years post-operatively. A study investigating telemetric data from a 42-year old patient with a distal femoral joint replacement compared the forces applied to the stem tip to those on the implant shaft. This data showed an increasing force at the stem tip over time with an increase in the stem tip-to-shaft force ratio of 25% to 63% over 23 months (Taylor et al., 1997). Supporting histological analysis of growing patients with extendible prostheses, shows bone remodelling adjacent to prostheses near porotic bone, and the growth of fibrous tissue under the implant shoulder. However, it must also be noted that the authors mentioned a possible effect on remodelling by the blood supply, which is interrupted during surgery and implantation of a cemented prosthesis. A subsequent ingrowth of blood vessels into necrotic bone from the invading fibrous tissue at the transection site occurs (Blunn and Wait, 1991). In addition, poor cement interdigitation, which is affected by the amount of cancellous bone and shape of the intramedullary canal into which the implant stem is inserted, could possibly affect the outcome of these prostheses (Unwin et al., 1996). This is an important point not only for massive implants but also during revision of femoral stems for THR as there is often sclerotic bone adjacent to the cement. Nevertheless, various methods have been employed to promote load transfer to the shaft of the prosthesis from the bone at the transection site in an attempt to prevent aseptic loosening. As the survival rate of patients is increasing, the long-term fixation of massive endoprostheses is of even

greater importance and it is possible that bone marrow stromal cells (BMSC) could play a role in biologically improving the fixation of these implants.

Bone is dynamic, and responds to compressive and tensile forces by bone formation and resorption respectively. Wolff's law, as first described by Julius Wolff in 1892, is the theory that mechanical stress determines bone structure, and that a change in stress can consequently result in changes in bone composition (Wolff 1892). Applying this theory to remodelling in cases of massive implants means that bone adjacent to massive implants change, because of the new compressive and tensile forces being applied. This is because of the redistribution of loads due to the modulus of elasticity of the metal used. Thus, certain trends in bone growth around these implants can be seen.

A common radiographic observation around massive implants, which may reflect the bending effect, is bone growth posteriorly and medially, whereas bone resorption is often seen anteriorly and laterally (Inglis and Walker, 1991); (Unwin et al., 1996). Inglis, et al. (1991) reported such a pattern of bone growth around massive proximal femoral replacements. In this survey, which analyzed both lateral and anteroposterior radiographs, it was postulated that cortical thickening in the areas experiencing compressive forces would form a "column" of bone, which would move down the implant with the pressure applied by adjacent muscles.

When prostheses are cemented, the consequence is that the host bone is under a decreased load and the strain is reduced, causing resorption. This phenomenon is known as "stress shielding" (Huiskes, 1990). This change in load distribution to the metal implant and reduction of load experienced by surrounding bone, causes strain adaptive remodelling to take place according to Wolff's law (Wolff 1892).

1.5 Hydroxyapatite for improving implant fixation

In some patients, a pedicle of bone grows from the transection site around massive metal implants, often with an intervening fibrous tissue layer between the implant and new bone (Blunn and Wait, 1991). Integration of the implant into the host bone by ingrowth and attachment of this new bone is thought to promote better load transfer to the shaft rather than the stem tip preventing "underloading" (Inglis and Walker, 1991) of the collar and overloading of the cement mantle (Okada et al., 1988); (Sim and Chao, 1979). One method that promotes this is the use of a collar on the implant shaft, at the transection site. These collars encourage bone to grow over and attach to the shaft of the prosthesis. Modern implant coatings try to augment the "bony bridge" by using porous titanium beads, bone graft, grooves and/or a hydroxyapatite coating (Blunn et al., 2000); (Chao and Sim, 1985), (Okada et al., 1988); (Ward et al., 1993).

Hydroxyapatite (HA) is a biocompatible, resorbable, osteoconductive material that has been found to greatly enhance bone growth in both patients and animal models (Blunn et al., 2000); (Coathup et al., 2000); (D'Antonio et al., 1992); (Geesink, 1993). Osteoconductivity is the ability of a scaffold to promote osteoblast attachment and bone formation. In comparison, an osteoinductive scaffold encourages osteoblast precursor cells, such as BMSC, to attach and differentiate into active osteoblasts. Natural bone is an example of an osteoinductive material (Datta et al., 2006). Another property of scaffolds, osteogenicity, refers to the presence of osteoblasts or osteoblast precursor cells that deposit bone directly on the scaffold surface (Mizutani et al., 1990).

Synthetic hydroxyapatite is a calcium phosphate ceramic that shares a similar chemical and crystallographic structure to natural hydroxyapatite, which is the main mineral component of bone (Engfeldt et al., 1953); (Jarcho, 1981). Although it has poor fatigue

properties, which prevents it from being a useful structural implant material, HA can be applied to metal surfaces using a plasma-spray technique. This provides an implant with osteoconductive properties while maintaining the mechanical properties of a metal implant (Geesink et al., 1988). The bonding of bone to HA-coated implants has been investigated previously in canines and was found to be very strong as a result of direct bonding of the material to bone (Geesink et al., 1988). As the HA is resorbed, released calcium and phosphate ions may also promote formation of a biological apatite layer, and thus promote osteoinduction (Kurioka et al., 1999); (Ozawa and Kasugai, 1996). However, concerns do exist about the stability of the metal implant-HA bond, in that it is non-permanent and may lead to wear particle production (Bloebaum et al., 1994).

The use of grooved, porous, hydroxyapatite-coated collars has shown effective results in terms of bone ingrowth to the implant collars (Blunn et al., 2000). In the short-term follow-up reported by the authors (after more than 12 months), only one patient out of forty-four showed any signs of loosening, and a radiographic study showed no signs of radiolucency around the implant (Chao et al., 2004) and significantly improved extracortical bone growth around massive implants in patients by using a porous-coated collar augmented with autologous bone graft. Only one out of forty-three patients showed aseptic loosening after a mean of 9.7 years follow-up. The use of cementless fixation with proximal femoral components showed that the use of HA coincided with a more even distribution of bone growth around the implant when compared with control implants (Blunn et al., 2000).

Compared to uncoated porous collars, hydroxyapatite-coated collars on distal femoral replacements appear to promote bone ingrowth in over 70% of cases. This ingrowth is negatively correlated to the extent of radiolucent lines on radiographs. Good bone formation into the HA-coated collars resulted in a decrease in the size and number of radiolucent lines around the intramedullary stem (Unwin, 2005), (Cobb, 1991).

As this uncoupling of load at the implant shoulder to the stem tip ultimately results in debonding and loosening of the implant (Unwin et al., 1996), strategies to prevent this initial uncoupling have been considered. Ideally, the use of a biocompatible material with an elastic modulus closer to that of cortical bone would prevent overloading of the stem tip. Recently, studies that have investigated more flexible materials have shown them to promote higher proximal shear stresses between the stem/cement and bone interface. This could result in debonding, micro-motion, and eventual loosening of the implant (Huiskes, 1990).

One strategy to reduce aseptic loosening (Figure 1.1) has been to improve the fixation of the implant to the bone by improving the osseous integration of the implant. This is thought to prevent the uncoupling of load from the implant shoulder through load transfer. This strategy has focused on promoting bone growth over the implant by developing implant collars at the transaction site of the implant. The resultant "bony bridging" is also thought to act as a "purse string," sealing off the bone-implant interface, possibly preventing the migration of wear particles to the site causing osteolysis. However, it should be noted that the existence of these wear particles has not been proved empirically, unlike the polyethylene wear particles generated with traditional arthroplasty.

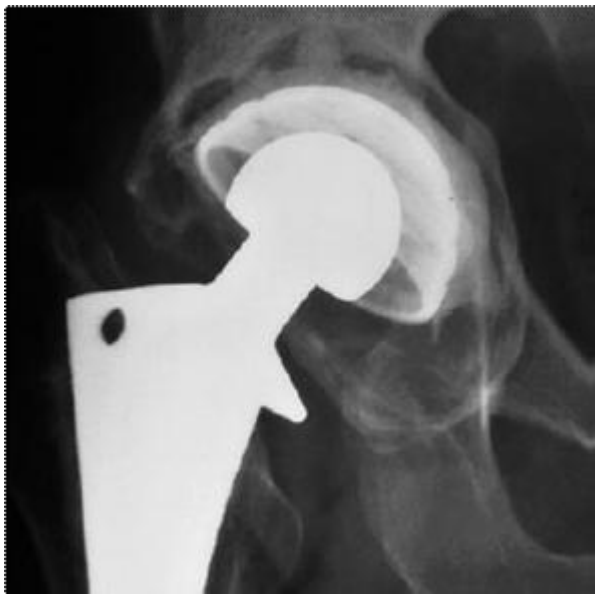


Figure 1.1 This figure shows a total hip arthroplasty. Lysis is seen around the acetabulum. Improving the cup bone osseointegration provides a biological bridge preventing the migration of lysis particles to the cup bone interface.

Another method by which fixation of the implant could be improved is by using osteoblastic precursor cells such as bone marrow stromal cells (BMSC) in combination with HA-coated collars to further encourage greater bone growth and attachment into the implant collar. To augment this beneficial load transfer and reduce the aseptic loosening of massive implants using BMSC is the goal of this thesis.

1.6 Stem cells

1.6.1 Definition

The term "stem cell" refers to an undifferentiated precursor cell that has "the capacity for unlimited or prolonged self-renewal, that can produce at least one type of highly differentiated descendant" (Watt and Hogan, 2000). These precursor cells can be induced to differentiate into another cell type, depending on the type of stem cell and

the cues given (Jaiswal et al., 1997). Between a fully multipotent stem cell and a terminally differentiated cell type, there are thought to exist a series of "committed progenitors" with a lower proliferative and differentiation potential. These can be referred to as "transit amplifying cells (Watt and Hogan, 2000).

There are many sources of stem cells cited in the literature: from the embryo, the adult bone marrow, umbilical cord blood, adipose tissue, as well as neuronal-derived cells (Bacou et al., 2004), (Lam et al., 2001); (Zvaifler et al., 2000); (Zandstra and Nagy, 2001), all of which have varying abilities to differentiate down numerous cell lineages. As promising as these cell sources may be, most past and current research concerns the first three types of stem cell. It is becoming increasingly apparent however, that adult stem cells isolated from the bone marrow have potential regenerative applications (Le Blanc and Pittenger, 2005), such as the tissue engineering of bone (Arinzeh et al., 2003), (Lee et al., 2005).

Autologous, Allogeneic and Xenogenic Cells, and the Multipotency of stem cells:

Definitions: In cases where the stem cells from a patient are re-implanted into the same individual, the donor cells are referred to as being autologous. If donor cells are implanted into another individual of the same species, the cells are termed allogeneic, and of another species, xenogenic. A totipotent cell has the ability to differentiate into any type of cell and this is usually the property of embryonic stem cells. Pluripotent cells can differentiate into almost all cell types, but not all. A cell that can only differentiate into a limited number of cell types is termed multipotent (Zandstra and Nagy, 2001).

1.6.2 Bone Marrow Stromal Cells (BMSC)

Bone marrow stromal cells (BMSC) are a population of plastic-adherent (capacity for adherence and growth on tissue culture plastic) cells derived from the bone marrow, that most often possess a fibroblastic morphology. BMSC, described as "fibroblastic precursor cells," were first described by Friedenstein, Chailakhjan and Lalykina in 1970 (Friedenstein et al., 1970). A population of cells within this heterogeneous population (Aubin, 1998) are commonly referred to as "mesenchymal" stem cells, referring to their multi-potentency and/or ability to form mesodermal tissues such as bone (Haynesworth et al., 1992); (Jaiswal et al., 1997), cartilage (Johnstone et al., 1998) and adipose tissue (Ryden et al., 2003), and stroma (Majumdar et al., 2000). Friendenstein's seminal work investigated the properties of bone marrow stromal cells, *in vitro*, by observing their ability to mineralise after treatment with osteogenic supplements, and by their ability to form bony tissue in diffusion chambers invivo (Friedenstein et al., 1970); (Friedenstein et al., 1976); (Friedenstein et al., 1968). Bab, Ashton, et al. (1986) also investigated BMSC in diffusion chambers, and found that the tissues produced in these chambers originated from a small number of originally implanted cells with "stem cell-like" characteristics. Friedenstein proposed a hypotheses leading from his work that suggested a single stromal cell could give rise to multiple lines of cells (Friedenstein, 1980). From their experimental data of CFU-F derived from the bone marrow, Owen, Cave, et al. (1987) produced a summary flow chart which indicated that stromal "stem cells" could differentiate into committed progenitors, which could then further differentiate into specific cell types such as fibroblasts, reticular cells, adipocytes, and osteoblasts. Dahir, et al. (2000) isolated "precursor" cell lines from mouse bone marrow and was able to show by labelling cells with the enzyme 3-galactosidase (using a non-infections retrovirus containing the lacZ gene), that these cells not only had osteogenic characteristics *in vitro*, and were able to repopulate the bone marrow of host

mice, they maintained their osteogenic capability.

However, there is some controversy as to whether or not BMSC are actually 'stem cells' as they are only multi-potent or have restricted differentiation potential, as opposed to embryonic stem cells (ESCs) which have the capacity to differentiate into almost any cell type (Heins et al., 2004); (Thomson et al., 1998). For this reason, the population of mononuclear, plastic-adherent, fibroblastic cells isolated from the bone marrow will be identified in this thesis as bone marrow stromal cells (BMSC), a term that refers to the cells origin and morphology.

In culture, these cells are selected from the marrow based on the following characteristics: their mononuclear phenotype, their capacity for adherence and growth on tissue-culture plastic and their ability to form fibroblastic colony units (colony-forming units-fibroblastic (CSFU- F). BMSC populations can also be enriched for "multipotent" cells or "stem cells," by selecting cells based on their surface marker expression pattern using fluorescence-activated cell sorter (FACS) or magnetic-activated cell sorter (MACS) machines (Baksh et al., 2003); (Campagnoli et al., 2001); (Pittenger et al., 1999); (Watt and Hogan, 2000).

Listed below (Table 1.1) are a panel of cell surface markers used in BMSC identification, some of which are expected to be present and others absent.

Surface markers present on BMSC	CD29, CD44, CD54, CD71, CD90, CD120a, CD124, CD105 (Endoglyn), CD106 (VCAM-1), CD164 (Endolyn), SH2, SH3, SH4, Stro-1, P-ZR, (P zero protein)
Surface markers absent on BMSC	CD14, CD34, CD45, CD68, HLA-DR

Table 1.1 Table illustrating cells surface markers present and absent on human BMSC (Baksh et al., 2003); (Campagnoli et al., 2001); (Pittenger et al., 1999); (Watt and Hogan, 2000).

BMSC have great promise for tissue repair and regeneration, due to their ease of isolation, high proliferative capacity and ability to retain their multi-potent capacity, as well as their ability to differentiate into various mesenchymal tissues. In addition, the immuno-modulatory effects of BMSC should also be considered as this may permit the use of allogenic cells in patients.

1.6.3 Previous characterisation of BMSC

In current practice, the positive identification of BMSC as multipotent precursor cells can be achieved through two methods: differentiation of the cells down two or more lineages, and surface marker identification. Ideally, both methods should be utilised for complete characterization.

Upon stimulation in culture with osteogenic reagents, BMSC change morphology and begin to express osteoblastic charactenstics temporally in the following order: cbfa-l/runx2 and osterix transcription factors involved in osteoblast differentiation; alkaline

phosphatase which is expressed on the cell surface of osteoblasts; osteonectin, which regulates mineral- collagen interactions and calcium binding; osteopontin, involved in calcium deposition; and osteocalcin, which regulates the later stages of mineral deposition (Krishnan et al., 2003); (Pittenger et al., 1999). When differentiated towards the chondrogenic lineage, BMSC seem to upregulate the expression of collagen II (basic matrix molecule of articular cartilage), aggrecan, and sox-9, in addition to a chondrocytic phenotype (Lee et al., 2006); (Mackay et al., 1998). After treatment with adipogenic reagents, BMSC appear to accumulate lipid vesicles and are positive for the expression of genes such as lipoprotein lipase (LPL) and nuclear peroxisome proliferators-activated receptor gamma (PPAR72) (Pittenger et al., 1999).

There has also been some evidence of neural and hepatocytic (Kang et al., 2005); (Lee et al., 2004) phenotypes being derived from BMSC, although this has also been disputed (Bertani et al., 2005); (Wang et al., 2003). Other groups have documented the differentiation of BMSC into cardiomyocytes and vascular cells (Nagaya et al., 2005). One group tracked cells and found that the implanted BMSC, in addition to releasing mitogenic, angiogenic, and anti- apoptotic factors *in vitro*, were positive for many cardiac markers once implanted into myocardia *in vivo*.

As previously mentioned, human BMSC (hBMSC) can also be identified through the presence and absence of a series of cell surface markers, none of which are specific to BMSC (Baksh et al., 2003); (Campagnoli et al., 2001); (Pittenger et al., 1999). Recent research has identified the cell surface proteins CD164/endolyn and a specific isoform of P zero-related (PZR) protein as being markers of hBMSC (Watt and Hogan, 2000), albeit they are not entirely specific. There has been some preliminary work looking at the gene expression of BMSC that seems to also indicate that the patterns of gene expression of BMSC are unique (Djouad et al., 2005); however, this is not yet a commonly available tool for most laboratories. Whether or not all of these markers are

present or absent from the BMSC derived from other species is unknown.

BMSC from other species have been isolated and either partially or fully characterised, such as from mice (Dennis and Charbord, 2002); (Phinney et al., 1999); rats (Lee et al., 2005); (Okamoto et al., 2006), pigs (Bosch et al., 2006); (Vacanti et al., 2005), cats (Martin et al., 2002), dogs (Kadiyala et al., 1997); (Volk et al., 2005), goats (Kruyt et al., 2003), sheep (Kon et al., 2000); (Shang et al., 2001), cows (Troyer et al., 2003) and baboons (Bartholomew et al., 2001).

Although Jiang, et al. (2002)were able to isolate a special population of cells from human, mouse and rat BMSC, called multipotent adult progenitor cells (MAPCs) that appeared to have pluripotency similar to ESCs, some of the FACS analysis for cells surface markers has been called into question (Verfaille, 2006), (Jiang, 2007) challenging the validity of the groups results.

1.6.4 Proliferation rate of BMSC

Human BMSC have been documented as having a high proliferation rate and can undergo as many as 38 population doublings without loss of their proliferative or osteogenic capacity (Bruder et al., 1997). This is almost twice the doubling capacity of other human diploid cell types, such as adult lung fibroblasts, which have a doubling capacity of 20 (Hayflick, 1965). With such prolonged expansion of BMSC in culture, there are accompanying concerns regarding cell ageing and mutation. Unlike undifferentiated embryonic stem cells [ESCs: (Heins et al., 2004)], BMSC do not express high levels of telomerase, the enzyme that legthens telomeres to prevent their shortening during cell division. As telomere shortening is directly related to cell ageing and senescence, there is a possibility that BMSC expanded in culture could lose their characteristics and ability to proliferate and differentiate once re-implanted *in vivo*

(Baxter et al., 2004). Another factor is an accumulation of mutations by the cells while in culture. It is well documented that not only immortalised tumour cell lines, but also BMSC cell lines could potentially mutate in artificial culture conditions (Devine et al., 2001). This may result in errors in cell division or DNA repair, which lead to translocations (movement of one segment of a chromosome to another) as well as aneuploidy (Wang et al., 2005), which can be defined as having an abnormal number of chromosomes. In addition, BMSC-like cells have been isolated from giant cell bone tumours (Wulling et al., 2003). The possibility of accumulated cell damage and ageing should, therefore, be seriously considered when investigating BMSC and thorough testing should be in place to ensure that such potentially tumourogenic mutations have not occurred before re-implantation or infusion of cells.

1.6.5 Immunogenetic properties of BMSC

Current research suggests that BMSC have suppressive immunogenetic properties. The first evidence of an immunosuppressive effect of BMSC was their ability to promote recovery and engraftment of Haematopoietic Stem Cells (HSCs) in high-dose chemotherapy patients receiving HSC infusions (Koc et al., 2000). A number of *in vitro* experiments have assayed the effects of BMSC on mixed lymphoyte cultures (Le Blanc et al., 2003); (Tse et al., 2003). Le Blanc, et al. (2003) added different concentrations of both autologous and allogenic human BMSC to mixed lymphocyte cultures in an attempt to measure immunoreactivity of the BMSC. Not only did the group observe a cell-dosage effect of BMSC on the proliferation of lymphocytes and that BMSC were able to prevent the formation of activated T-lymphocytes, they also found that this effect was wholly independent of the major histocompatibility complex (MHC). Rasmusson, et al. (2003) obtained supporting results in a similar study.

Further work demonstrated that BMSC retained their immuno-suppressive properties after *in vitro* differentiation into osteogenic, chondrogenic, and adipogenic cells (Le Blanc et al., 2003). What is the mechanism of this immunomodulatory effect? BMSC express MHC I and lymphocyte function-associated antigen (LFA)-3 antigens as well as MHC II and ICAM-I antigens after stimulation with γ-interferon treatment (Tse et al., 2003), but the observed effects are independent of the MHC. Evidence by Aggarwal and Pittenger (2005) showed that prostaglandin E2 (PGE2) plays a substantial role in mediating anti-inflammatory and immunomodulatory responses by showing a reversal of the immunosuppression after treatment with a known PGE inhibitor. It must be noted, however, that an earlier experiment by Tse et al. (2003) found that inhibiting PGE, had no such effect. The mechanism of BMSC-modulated immunosuppression has not been conclusively studied as yet, however, this property may explain the acceptance and effectiveness of allogeneic cells in a few bone tissue engineering models (Tsuchida et al., 2003) (Arinzeh et al., 2003); (De Kok et al., 2003) . In these models, allogeneic BMSC have performed as well as autologous BMSC in regenerating defects with new bone.

1.6.6 Haematopoietic Stem Cells (HSCs)

HSCs are precursors of the haematopoietic lineage, which are predominantly localised to the bone marrow in their undifferentiated state, and most often derived from this source. Till and McCulloch (1980) first documented the multipotency of these bone marrow-derived cells after an injection of HSCs into lethally irradiated mice resulted in the formation of donor-derived haematopoietic colonies of various lineages. In comparison to BMSC, HSCs are well documented in terms of their differentiation pathways and mechanisms, localisation and attachment in the marrow, and the clinical

results from transplanting these cells into human patients. Donor HSC infusions have proven to be clinically effective in the treatment of neutropenia (Reddy, 2005). This often involves a selection of cells expressing the cell surface marker CD34 using fluorescence-activated cell sorting (FACS) from a plasma-depleted blood donation, followed by infusion of these cells into the patient. Clinically HSCs are often mobilised using granulocyte-colony stimulating factor (G-CSF) or granulocyte-macrophage-colony stimulating factor (GM-CSF). The mobilisation of these cells is mediated via the binding of HSCs to the bone marrow stroma. Upon stimulation, the HSC cell surface receptor CXCR4 dissociates from the cytokine stromal-cell derived factor-1 (SDF-1), a factor expressed by BMSC and other cells of the stromal lineage. A second mechanism involves the release of the HSC adhesion molecule VLA-4 from vascular cell adhesion molecule-1 (VCAM-1), which is expressed on the surface of BMSC. In both cases, there is liberation of proteases such as elastase, cathepsin G and matrix metalloproteases (MMPs) such as MMP-9, which result in the degradation of these molecules involved in adhesion as well as a subsequent remodelling of the ECM microenviromnent to promote release of cells from the marrow (Lapidot and Petit, 2002).

1.6.7 Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are isolated from the inner cell mass of pre-implantation. ESCs were first isolated from mice by Martin Evans in 1981 (Evans and Kaufman, 1981). Human embryonic stem cells were first successfully isolated and cultured into cell lines in 1998 by (Thomson et al., 1998). ESCs are pluripotent, as they can differentiate into almost any cell type except extra-embryonic cell types such as the yolk sac visceral endoderm, parietal endoderm, and placental trophoblast (Zandstra and Nagy, 2001).

The advantage of using ESCs is their ability to differentiate into any tissue derived from the three embyonic lineages (ectoderm, mesodenn, and endoderm) in two or three-dimension culture. Also ESCs hold great promise in the pharmaceutical industry for drug discovery and toxicity testing. At present, there are insufficient sources of specialised human cell types such as those derived from the liver or heart (hepatocytes or cardiomyocytes). By differentiating the highly proliferative, pluripotent ESCs into these cell types, new cells would be available for *in vitro* testing. In fact, as discussed by (Ameen et al., 2008) these cells could even be used to derive cells that could activate and mobilise endogenous cells to organs or tissues in need of repair or improved function.

The potential disadvantages of using ESCs are numerous, and include the difficulties and expense associated with ESCs (Le Blanc and Pittenger, 2005). It is impossible to use autologous ESCs, which are derived from embryos, as there are immunoreactivity issues to be considered in transplanting human leukocyte antigen (HLA)-mismatched cells into patients (Heng et al., 2005). When using BMSC, it is possible to use autologous cells from the patient, expand those cells, and re-implant them without any donor-related risks. Another disadvantage is the difficulty in culturing ESCs. Although ESCs can be grown and expanded *in vitro,* if they not maintained under strict control, the cells spontaneously differentiate into different cells, some of which may not be the desired type (Heng et al., 2005). For example, a study investigating ESCs administered *in vivo* in immune-compromised mice Tzukerman et al. (2003) showed that ESCs can give rise to teratomas and are tumourogenic. However, if ESCs were pre-differentiated into specific cell types before implantation, the probability of terartomic activity could decrease significantly (Ameen et al., 2008). These factors have limited the potential of ESCs for clinical application. The third, and perhaps most controversial consideration in using ESCs, is the ethical and legal dilemma posed by their potential use clinically. An

important source of human ESCs is embryos created through *in vitro* fertilisation procedures. The moral idea that embryos have the potential to become human beings and, therefore, should not be dissected for use in research, and/or clinical practice, has made the use of human embryos both difficult practically and ethically controversial (Shenfield, 2005); (Young, 2000). When mesenchymal tissues are regenerated in tissue engineering, adult stem cells such as BMSC have been preferred as they are readily available, can be isolated from the patient who could be receiving therapy (as autologous cells), and have the potential to differentiate into the tissue of interest.

1.7 Tissue engineering

1.7.1 Introduction

Organ and tissue transplants have been used for some time in clinical practice in many areas of medicine with varying levels of success. Supply and demand as well as immunocompatibility and disease transmission are big issues in transplantation that often render them impossible or risky. Immunosuppressive drugs taken to accept these organ transplants can lead to tumour formation (Vacanti et al., 2000). By creating tissues using the basic components of cells, scaffolds, and bioactive factors, tissue engineering attempts to replace, re-construct, or repair tissues. There are two main approaches in this field, which are *in vitro* (or *ex vivo*) and *in vivo* tissue engineering. *In vitro* tissue engineering strives to create tissue outside the body. This may require the use of tissue culture techniques, in addition to bioreactors, bioactive factors, and specially designed scaffolds and biomaterials.

1.7.2 Scaffolds

One of the important components of tissues in the body, apart from the cells themselves, is the extracellular matrix (ECM) that they lay down. ECM serves a variety of functions, from general structural support of the tissue, to involvement in cell alignment and communication (Petreaca et al., 2007). This matrix is predominantly collagen-based, although other molecules such as hyaluronic acid, glycoproteins, elastins, fibronectin, vitronectin and laminin are also important components (Pearson et al., 2002). Embedded within the fibrils of these matrix materials, various growth factors can be found as well as water molecules and enzymes such as elastases or metalloproteases (Petreaca et al., 2007). In tissue engineering, scaffolds attempt to simulate a native tissue environment with an artificial extracellular matrix. Ideally, the material should also be biodegradable (Pachence et al., 2000). Thus, popular scaffolds used include collagen I, alginate, poly-lactic acid (PLA), poly-glycolic acid (PGA), plasma, as well as fibronectin and fibrin (Andree et al., 2001); (Gurevich et al., 2002); (King et al., 2006); (Kumar and Albala, 2001); (Pachence et al., 2000); (Ting et al., 1998); (Ye et al., 2000).

In bone tissue engineering, a mechanically supportive, osteoconductive, ceramic scaffold such as natural coral exoskeleton, synthetic hydroxyapatite, beta-calcium triphosphate or Bioglass may be used (Chan et al., 2002); (Oonishi et al., 1997), often in combination with one of the above mentioned matrix materials. These scaffolds have inherent osteoinductive (Ripamonti, 1996); (Yuan et al., 1998) and/or osteoconductive properties (Geesink and Hoefnagels, 1995). Newly developed biomaterials include silk-based scaffolds (Meinel et al., 2005) and chitin-based scaffolds (Di Martino et al., 2005), which appear to be osteoconductive *in vivo*. BMSC are generally combined with one or a combination of these scaffolds with or without growth factors, for bone tissue engineering (Bruder et al., 2000).

Porosity and pore sizes have been investigated for their effects on bone regeneration with scaffolds, with or without cells being added. Various works report that, overall, the minimum pore size of a structure should be greater than 300 μm, to allow for cell migration into the, pores, nutrient migration, as well as to allow the invasion of capillaries into the site; vasculogenesis being an important first step towards osteogenesis (Kuboki et al., 2001); (Tsuruga et al., 1997); (Gotz et al., 2004). While smaller pores can contribute to hypoxic conditions and the aggregation of cells, resulting in osteochondral ossification, larger pores allow for direct osteogenesis. Although higher scaffold porosity encourages better bone in-growth, increasing porosity often decreases the mechanical strength of a scaffold, to the point where it is no longer withstanding the loads seen in orthopaedic applications (Karageorgiou and Kaplan, 2005).

1.7.3 Fibrin Glue

Fibrin is a naturally occurring matrix which has an important role in the final steps of the coagulation cascade in wound healing, and has been used previously as a tissue engineering scaffold (Grant et al., 2002); (Lee et al., 2005). In the body, fibrin is formed by the enzymatic cleavage of the fibrin zymogen fibrinogen by the serine protease thrombin. Fibrin then undergoes a polymerisation reaction with other fibrin molecules, as well as cross-linking (facilitated through either Factor XIII or endogenous trans-glutaminases) to itself and surrounding tissue, which lends it both haemostatic and adhesive properties (Marx and Mou, 2002). Fibrinogen fragments as well as fibrin degradation products are thought to promote the migration of cells such as fibroblasts (Abiraman et al., 2002) and vascular smooth muscle cells to the healing site (Kodama et al., 2002). Fibrinogen-fibroblast interactions have not been thoroughly investigated

(Gailit et al., 1997). Fibroblasts are thought to interact with fibrin by the interaction of fibrinogen molecules with the integrins œyßi, œyfis, œiibßs (Jeffrey A et al., 2007). Integrins are a large family of cell surface receptors involved in adhesion by binding to ligands found expressed in the ECM and by other cells (Gailit et al., 1997). Although there is evidence for direct fibroblast-fibrinogen interactions in cell adhesion, evidence shows that indirect interactions with fibronectin are required for cell spreading and migration into fibrin via the crucial integrin binding to the sequence RGDS (Gailit et al., 1997); (Knox et al., 1986); (Pierschbacher and Ruoslahti, 1984).

Cells naturally express urokinase plasminogen activator and tissue plasminogen activator, serine proteases that activate the fibrin-degradation enzyme plasmin (Neuss et al., 2004), and this process, in addition to the action of macrophages and multi-nucleated foreign body cells, naturally degrades fibrin over time.

Fibrin glue has been used for many years as a surgical haemostatic agent (Davidson et al., 2000); (Spotnitz et al., 1987), as well as other surgical uses, for example, preventing air leaks after pulmonary resection (Belboul et al., 2004). Fibrin glue can also be sprayed. Commercially prepared fibrin glue, such as Tissucol® and Tisseel® fibrin sealant, have two main components: human fibrinogen reconstituted in a bovine aprotinin solution, and a human thrombin-calcium chloride solution. The availability of fibrin, in addition to its simple application system, makes it easy to obtain and use. Combinations of fibrin and non-resorbable HA have been used throughout Europe for over a decade, in the reconstruction of maxillofacial and dental defects (Bonucci et al., 1997). The fibrin phase has proved effective in moulding ceramic scaffolds and holding the granules/powder in place as natural mineral deposition occurs.

At present, fibrin is a promising scaffold for cells for skin regeneration, and when combined with keratinocytes, can be sprayed onto wound sites (Grant et al., 2002).

Fibrin has been shown to increase the level of the angiogenic factor VEGF, which could enhance the healing effect of fibrin scaffolds in skin tissue engineering (Hojo et al., 2003).

The osteoconductive properties of fibrin glue have been reported (Abiraman et al., 2002); (Bosch et al., 1980); (Kania et al., 1998); (Yamada et al., 2003) and disputed (Greco et al., 1988); (Lucht et al., 1986); (Schwarz et al., 1993). An experiment by Abiraman, et al. (2002) showed that fibrin glue had osteoconductive properties at an ectopic site; however, this was only observed when fibrin was combined with hydroxyapatite or bioglass. Similar work in a rat femoral defect model indicated a role for fibrin glue in osteogenesis when implanted in an orthotopic site. In another study by Schwarz, et al. (1993) fibrin glue did not promote osteoinduction at both ectopic and orthotopic locations in rats. Thus, no study has shown fibrin alone to possess osteoinductive properties.

Fibrin may not induce differentiation of osteoblastic precursors into osteoblasts, but may promote neo-vascularisation at the surgical site to promote faster healing and bone formation and organisation (Kania et al., 1998). In the osteoconductive experiments described above, control biomaterials such as bioglass and hydroxyapatite granules did not show reduced bone formation and bone ingrowth to materials when compared to those treated with fibrin, but also lacked neo-angiogenesis (Kania et al., 1998). A study by Abiraman, et al. (2002) demonstrated that the ectopic implants coated in fibrin glue had noticeable neo-vascularisation and evidence of intramembranous ossification when compared to controls, which were encapsulated in a thick fibrous layer.

Fibrin's biocompatibility, commercial availability, in addition to its proven effectiveness in bone defects, makes it an ideal cell delivery vehicle for cells such as BMSC in *in vivo* tissue engineering adaptations.

1.7.4 The use of BMSC in the tissue engineering of bone

The first evidence indicating the osteoinductive nature of bone marrow cells was the ability of fresh bone marrow to augment bone formation in orthopaedic surgery (Boyne and Yeager, 1969); (Morris, 1969). Following the seminal work of Friedenstein, et al. (1968) into the role of BMSC specifically in bone formation, they have become a major component of bone tissue engineering strategies. One benefit of using BMSC as opposed to bone marrow is the lack of erythrocytes, which may increase potassium to cytotoxic levels (Street et al., 2000). In vitro testing has frequently been used as a pre-indicator of osteogenic capabilities of osteoprogenitor cells and cell-scaffold costructs in vivo (Jaiswal et al., 1997), (Rust, 2004). BMSC have been shown to increase bone growth in vivo in small and large animal models at both orthotopic and ectopic locations (Bruder et al., 2000); (Haynesworth et al., 1992), (Kalia et al., 2006); (Korda et al., 2006); (Kruyt et al., 2003). Some of the first experiments investigating the potential of BMSC in *in vivo*, loaded cubes of calcium phosphate ceramics with or without BMSC, which were implanted subcutaneously in synergenic or immuno-compromised rodents (Haynesworth et al., 1992). These studies found significantly increased bone growth into the cubes loaded with BMSC when compared to the scaffolds alone. Further work by this group showed that this observation was applicable to cells from many mammalian species, such as goats, sheep, dogs, rats, and humans. Additionally, it was found that diluting the number BMSC up to 50% with fibroblasts did not effect bone formation *in vivo* (Bruder et al., 2000). In addition to smaller animals such as mice, rats, and rabbits, larger animals such as dogs, sheep, and goats, are often used for the *in vivo* testing of bone tissue engineering strategies. The results of *in vivo* investigations on the mechanical loading of the hind limbs of large animals indicate similar loading levels to those experienced by humans (Bergmann et al., 1984); (Buma et al., 2004); (Martini et al., 2001), suggesting that large animals may be a

suitable model for the testing of tissue engineering models with BMSC. A study investigating ectopic bone growth in a caprine model showed a significant effect of BMSC loaded seven days pre-operatively or per-operatively onto porous ceramic cubes, suspended in autologous plasma, and implanted into the bilateral paraspinal muscles of goats (Kruyt et al., 2004). This biphasic calcium phosphate (BCP) scaffold has previously been shown to be osteoinductive, in that it promotes bone growth in an ectopic site, without the aid of osteoinductive growth factors or osteoprogenitor cells (Yuan et al., 2002). In the 2004 ectopic study by Kruyt, et al., a comparison was made between cryopreserved and fresh cells to see if cells could be obtained in advance of surgery and preserved for future use. These groups were also compared to control, untreated scaffolds as well as devitalized scaffolds. These are scaffolds which have cells grown onto them for seven days, after which the construct is devitalised to remove the cells, leaving behind an extracellular matrix coating. In this study, it was found that these ceramic blocks had significantly more bone growth when seeded pre-operatively or per-operatively with BMSC compared to the scaffold only or a devitalised cell matrix/scaffold combination.

Kon, et al. (2000) preceded their orthotopic, large animal work with a small animal study demonstrating the osteoinductive effects of ovine BMSC when loaded into fibrin-coated HA blocks, and implanted subcutaneously in nude mice. Interestingly, it has been suggested that an ectopic intramuscular site, which is extremely vascular, could be an effective *in vivo* bioreactor for bone tissue (Stevens et al., 2005); (Warnke et al., 2006).

Bone tissue engineering can also be applied to cases where bone has been resected to

treat tumours such as high-grade osteosarcomas, Ewing's sarcoma, or malignant fibrous histiocytoma (Kenan, 1991). The patients are given pre-operative and post-operative chemotherapy to prevent the spread of the tumour and the possibility of metastases. One study demonstrated an improvement in five-year survival of stage IIB osteosarcoma patients from 32 without chemotherapy to 57 with this treatment (Eckardt et al., 1991a). Lee, et al. (2005) showed a negative effect of a neoadjuvant chemotherapy regime on BMSC proliferation on osteogenesis *in vitro* and new bone formation in a rat osteotomy model, by using a combination of doxorubicin and cisplatin. However, he was also able to demonstrate in a rat femoral defect model with external fixation that the application of fibrin glue and BMSC to the defect site resulted in similar amounts of bone formation, whether or not the rats had been given chemotherapy. Therefore, both these studies indicate a role for BMSC in bone regeneration after chemotherapy, as well as the effectiveness of BMSC in combination with fibrin glue for augmenting new bone formation at an orthotopic site.

In 1998, Bruder, et al. demonstrated the effectiveness of autologous BMSC in the healing of canine segmental bone defects, when the cells were loaded onto a fibronectin-coated, ß-TCP cylindrical construct. The femoral implants were supported by a lengthening plate and left in situ for 16 weeks. In this case, the BMSC resulted in a noticeable bony callus around the implant, as well as significantly more bone formation than the controls (untreated with fibronectin and cell-free). Untreated defects (with no implant or treatment) resulted in non-unions after sixteen weeks, and those ceramic implants that were not cell-loaded began to show signs of cracking at twelve weeks, in contrast to the cell-loaded implants. This same group demonstrated similar results after 16 weeks in the same canine defect model, but this time substituting allogeneic BMSC that were dog leukocyte antigen (DLA, analogous to HLA) mismatched as opposed to

autologous cells (Arinzeh et al., 2003). Petite, et al. (2000) attempted critical-sized defect bone regeneration with the aim of preventing non-union. As an alternative to autograft, the group created a BMSC-coral "biohybrid," and compared bone formation and union of the scaffold to host bone in an ovine model. The group first established the minimum length for non-union in sheep by creating a series of metatarsal defects and observing their ability to achieve union, that is, ability to heal and form new bone to bridge the gap rather than become scarred (Petite et al., 2000). They then created a "biohybrid" to fit the critical-size defect and tested BMSC-loaded coral constructs and fresh bone marrow-loaded constructs to cell-free scaffold controls and untreated defects. They found that while the untreated defects did not heal after 16 weeks, cell-free scaffolds achieved some bone growth, although they did not integrate into the host bone. Those constructs containing fresh bone marrow were filled with fibrous tissue more akin to scar tissue than bone formation, whereas BMSC-loaded constructs demonstrated mostly new bone formation and union with the host bone (Petite et al., 2000).

In another large animal model, a BMSC-loaded construct was implanted into critically sized defects of sheep and BMSC were loaded into HA cylinders by coating them with fibrin and absorbing a cell solution into the fibrin-ceramic scaffold. This cell-loaded group had noticeably more bone growth into the construct, as well as greater callus formation around the implant bridging to the host bone (Kon et al., 2000). However, it must be noted that the group size was too small for meaningful statistical analysis. Similarly, for other types of tissue engineering of mesenchymal tissues such as articular cartilage and craniofacial tissue (Frosch et al., 2006); (Shang et al., 2001) ceramic or metal implants have been used as a scaffold for BMSC, and tested in large animal models. In these cases it has been shown that BMSC can be used to restore other tissues

that provide important functional and or aesthetic roles.

1.8 Growth factors: Cytokines to target bone formation and revasularisation

In an effort to stimulate natural molecular mechanisms that trigger bone formation, one can implant growth factors at a site where bone regeneration or formation is required, or incorporate them into a scaffold in the presence or absence of osteoprogenitor or osteoinductive cells. These factors can be used to target the temporal mechanism of bone growth. In this case bone morphogenetic proteins such as BMP-2, BMP-4, BMP-7 or BMP-9 can be used to promote fracture healing or healing of critical sized defects (Lin et al., 2005); (Mistry and Mikos, 2005). Genes that also have been demonstrated to promote bone formation include sonic hedgehog (Edwards et al., 2005) and Runx2 (Byers et al., 2006). Vascularisation can be promoted by using growth factors such as VEGF, whether by direct protein or gene (DNA) injection into a site of bone repair (Geiger et al., 2005); (Huang et al., 2005). There are, however, some disadvantages to using growth factors, such as their great expense, making regular use exorbitant. For example, a study by (Dimitriou et al., 2005) found that the average cost of treating a fracture non-union with BMP-7 was approximately £7,338. In an American study by the Washington State Department of Labour and Industries (2003), treating a tibial non-union with BMP-2 cost approximately $12,468 (about £6,764), which was about the same cost as treatment with autograft. Also, the effectiveness of growth factors is not completely predictable, and in some cases is comparative to the use of autograft (Friedlaender et al., 2001); (Maniscalco et al., 2002). Autograft in this scenario refers to bone graft harvested from another site on the patient, such as the iliac crest (Burkus et

al., 2003). Similarly, osteoprogenitor cells or other cell types used for tissue engineering purposes can be genetically modified to express these growth factors. Use of a temporary vector, such as replication-incompetent adenovirus, which will insert its DNA into host cells and express the protein for a limited period of time, has its advantages (Lieberman et al., 1999); (Tsuchida et al., 2003). These advantages include the targeted temporal expression of the genes of interest, as well as preventing over-expression of the gene, and not using a virus that inserts into nuclear DNA prevents the risk of adverse immune reactions or the formation of a recombination-competent virus. In some cases, the adenoviral vector has been injected directly into a site of repair (Kang et al., 2004).

However, long-term expression of growth factors by cells can be achieved by using retroviral vectors. Replication incompetent retroviruses have been used to insert genes into the host genome of cells being implanted for use in bone tissue engineering (Breitbart et al., 1999); (Mason et al., 2000).

1.9 Goals

The main hypothesis of this thesis is that viable BMSC can be applied to implants using a fibrin glue-spray system, and increase bone formation adjacent to the implants and improve bone-implant contact.

This work aimed to improve fixation of orthopaedic implants to host bone in an attempt to reduce rates of aseptic loosening, and can be divided into four main aims:

1) To demonstrate the ability of BMSC to improve implant fixation in a large animal using practical scenarios such as total hip replacement, massive endoprosthetic

replacement and bone defect around pins.

2) To study the effect of varying cell dosages of BMSC in their ability to produce new bone and improve bone implant contact.

3) To study the effect of differentiating the BMSC along the osteogenic pathway in their ability to produce new bone and improve bone implant contact.

4) To study the effect of using semi-permeable barriers around BMSC sprayed on implants to prevent cell migration.

Chapter 2

2. Isolation, Expansion, Differentiation and spraying of BMSC Suspended in Fibrin Glue onto the Surface of Implants

2.1 Introduction

In the first part of this chapter, I have discussed the process of isolation, expansion and characterisation of BMSC from iliac crest bone marrow aspirates. In the second part of this chapter, I have discussed the spraying technique we have developed to coat implant surfaces with BMSC. The techniques described here are modified from the earlier work undertaken in our research centre (Rust, 2004, Kalia, 2007). In my subsequent experiments described in Chapters 3, 4 and 5 in this thesis, I have followed the methodology described here for the isolation and expansion of cells and for spraying the BMSC on the surface of implants intra-operatively.

2.2 Isolation, Culture expansion and Characterization of BMSC

2.2.1 Aspiration of BMSC

2.2.2 Animal model

English mule ewes were used to test the massive endoprosthesis model and tibial pin model. Sannan goats were used for the total hip replacement model. These animals were skeletally mature (two to five years in age), and weighed between 65-90 kg. All procedures were carried out according to the Home Office Animals Scientific Procedures Act of 1986. I held a personal license for animal handling during the period for which the experiments were undertaken. The technique of bone marrow aspiration is described below. The process of preparing the animals for surgery explained here was

followed in all operative procedures described in this thesis.

2.2.3 Anaesthesia and Pre-Operative Preparation

All animals were brought into the preparation room from their pens on the day of surgery and their vital measurements (blood pressure, pulse, respiratory rate and temperature) were recorded. Per-anaesthetic medication in the form of an intramuscular (IM) injection of Xyalzine (0.1 mg/kg, Rompun, Bayer Health Care, Newbury, Berkshire, UK), was administered approximately 30 minutes prior to induction of anaesthesia. Anaesthesia was induced using a combination of intravenously administered Ketamine (2 mg/kg, Ketaset, Fort Dodge Animal Health Ltd, Southampton, UK) and Midazolam (2.5 mg, Hypnovel, Roche Products Ltd., Welwyn Garden City, Hertfordshire, UK) intravenously (IV) injected into the external jugular vein. This was followed by a rapid sequence endotracheal intubation and attachment to an anaesthetic machine. A mixture of 2% halothane (Merial Animal Health Ltd., Harlow, Essex, UK) and oxygen, delivered at an optimum titrated volume was used to maintain the anaesthesia throughout the procedure. Continuous monitoring of the respiratory and cardiovascular parameters was undertaken from the time of administering the pre-medication, until the animals completely recovered and were transferred back to their pens. A stomach tube was routinely inserted to allow continuous drainage during the procedure and prevent the risk of aspiration from the reflux of gastrointestinal contents.

2.2.4 Procedure for Bone Marrow Aspiration

Prior to transfer to the surgical procedure room, an approximate area of 10 cm^2 of the

skin overlying the iliac crest was prepared for the procedure by shaving and then scrubbing with an antiseptic solution Pevidine, a Povidone-iodine scrub antiseptic solution (C-Vet, Bury St. Edmunds, UK). A fenestrated drape was used to protect the prepared surgical site from contamination until the scrubbed surgeon undertook further skin preparation.

The surgical site was scrubbed using a 0.5w/v chlorohexidine gluconate solution in spirit (Adams Healthcare, Leeds, UK) as a sterilizing agent and the animal was draped with sterile towels, exposing only the desired site.

The bony prominence of the iliac crest was then palpated and a 1cm incision was positioned on top of the bone. A surgical artery clip was used to blunt-dissect the soft tissues between the skin and the bone. The intramedullary cavity of the iliac crest was penetrated with a commercially available Jamshidi biopsy needle (R56780, Rocket Medical, Washington, UK) using constant pressure and a screwing motion. Routinely, 8 mL of bone marrow was aspirated, in two separate 4 mL aliquots taken from a deeper or shallower site (Rust, 2004), (Kalia, 2007). The aspirate was collected in syringes pre loaded with 0.5 mL heparin (1000 units/mL) (Monoparin, CP Pharmaceuticals, Bamstable, UK) to prevent coagulation of the samples.

Post-operatively, all animals were given 0.6 mg Buprenorphine as an analgesic (Vetergesic, Alstoe Animal Health, Melton, Mowbray, UK), as well as a long-acting antibiotic (15 mg/kg Amoxillicin, Betamox LA, Norbrook Laboratories (GB) Ltd., Great Corby, Carlisle, UK).

2.2.5 BMSC Isolation and Culture Expansion

The components of bone marrow were separated using a sucrose gradient and density

centrifugation. The procedures were undertaken in a designated laboratory under a lamellar flow hood using universal safety precautions.

Four mL of bone marrow aspirate was carefully layered on top of 3 mL of Ficoll-Paque™ PLUS sucrose gradient (17-1440-02, Amersham Biosciences, Chalfont St. Giles, UK). The layered samples were centrifuged at a temperature of 4°C for 30 minutes at a velocity of 1500 rotations per minute (rpm). The centrifuge segregated two layers. The lower layer had a distinct upper 'buffy' portion separating it from the upper layer. This 'buffy layer' contains the mononuclear cells of the bone marrow (Ficoll® users' guide). The buffy layer was carefully transferred into a universal container and washed in a solution of 10 mL of DMEM media (DMEM+, or control media), supplemented with 10% fetal calf serum (FCS, First Link, UK) and l00 units/mL penicillin/streptomycin (P/S, 0082, Invitrogen, Paisley, UK) to form a cell suspension.

The cell suspension was then centrifuged at room temperature for 5 minutes at a velocity of 2000 rpm. The supernatant was discarded and the remaining pellet resuspended in 1 mL DMEM+ using a 21 gauge needle and 1 mL syringe before being transferred to a T25 polystyrene cell culture flask (Coming, Coming, NY, USA) with 4 mL of media. This flask has a surface area of 25 cm^2 for cell expansion, and a treated (using oxidizing chemicals) surface for cell adhesion (Ramsey et al., 1984), (Amstein and Hartman, 1975).

Flasks were stored in a humidified incubator at 37°C, with 5% CO_2. Over 7-14 days, the cells expanded over the surface of the flask and colony-forming units (CFU-Fs) appeared. Once 70% of the culture surface was covered with cells, (70% confluent), the following steps were undertaken to expand the cells further (known as 'passaging').

2.2.6 'Passaging' of BMSC and the 'Live-Dead' Assay

The culture media (DMEM+) was pipetted out and the cells were washed with cold phosphate buffered saline (PBS). After the excess PBS was pipetted out, a 0.5% trypsin solution [0.5% trypsin-5.3 mM EDTA-4Na solution (15400-054, Invitrogen, Paisley, UK)], prepared by diluting a 10x stock solution with PBS was added to the cells in the culture flask. The culture flask was incubated for 5 minutes at 37°C. The trypsin lifts the cells off the culture surface. After 5 minutes, a 1:1 volume of DMEM+ is added to the culture flask to neutralize the effect of trypsin.

In order to determine cell viability, a 0.4% Trypan Blue solution (Sigma-Aldrich, Dorset, UK) was then used in a 1:1 ratio with a small quantity of the cell suspension. A haemocytometer was used to quantify the concentration of cells under a phase-contrast light microscope. Trypan Blue is a dye with two azo chromophores. The chromaphore is negatively charged, and thus, only reacts with cells when the membrane is damaged. Therefore, it is assumed that the (blue) stained cells are dead and the unstained (clear) cells are alive (Biosource AlamarBlue™ manual). The cell suspension was then centrifuged for 5 minutes at 2000 rpm at room temperature to obtain a cell pellet. The supernatant was discarded and the cell pellet was then resuspended in 1 mL of DMEM+ (control media) using a 21 gauge needle and syringe. Approximately 3,000 - 4,000 cells were seeded per cm^2 of cell culture area and cells cultured in 0.1333 mL of DMEM+ (control media) per cm^2 of surface area. DMEM+ (control media) media was changed every 3-5 days. This technique was used to expand cells when they reached 70-80% confluence on the cell culture flasks, until passage 3-4 at which time they were used in *in vitro* and *in vivo* experiments.

2.2.7 Differentiation of Bone Marrow Stromal Cells

2.2.8 Osteogenic Differentiation

Osteogenic Media (Rust, 2004), (Kalia, 2007).

Dulbecco's Modified Eagle's Medium (DMEM) (4500 mg/L glucose, D6429, Sigma-Aldrich, Dorset, UK)

10% Fetal calf serum (FCS; First Link, UK)

1% Penicillin/streptomycin (P/S; 0082, Invitrogen, Paisley, UK)

l.0 x 10^{-7} M Dexamethasone, water-soluble (D2915, Sigma-Aldrich, Dorset, UK)

5.0 x 10^{-4} M Ascorbic Acid (255564, Sigma-Aldrich, Dorset UK)

1.0 x 10^{-2} M β-glycerophosphate (G9891, Sigma-Aldrich, Dorset, UK)

BMSC were isolated, expanded, and trypsinised as described above. For histochemical staining (Von Kossa staining), 6 x 10^4 cells were plated out onto Thermanox® coverslips (Nalge Nunc intemational. Rochester, NY, USA), which had been placed at the bottom of the wells of 12-well plates (Coming, NY, USA). 1 mL of supplemented osteogenic media was added to each well. Cells were grown in a standard humidified incubator, and media was changed every 3-5 days. Cell morphology was observed every 5-7 days, up to 28 days after the initiation of culture.

For the alkaline phosphatase (ALP) assay, 6.0 x 10^4 cells were plated into 6-well plates (Nunc. NJ, USA) and grown for 7, 14, 21 and 28 days. For each cell line, there were two groups: cells grown in standard supplemented media, and cells grown in osteogenic media. Each group was repeated in triplicate for each time point.

2.2.9 Adiopogenic differentiation

Adipogenic Media [from (Kalia, 2007), (Rust, 2004)]

Dulbecco's Modified Eagle's Medium (DMEM) (4500 mg/L glucose, D6429 Sigma-Aldrich, Dorset, UK)

10%	Fetal calf serum (FCS; First Link, UK)
1%	Penicillin/streptomycin (P/S; 0082, Invitrogen, Paisley, UK)
l.0 x 10^{-6} M	Dexamethasone, water-soluble (D2915, Sigma-Aldrich, Dorset, UK)
200µM	Indomethacin (I7378, Sigma-Aldrich, Dorset, UK)
500µM	1 -methyl-3-isobutylxanthine (15879, Sigma-Aldrich, Dorset, UK)
10µg/mL	Insulin (I0516, Sigma-Aldrich, Dorset, UK)

For adipogenesis, $1x10^5$ cells were cultured per well in 12-well plates, on Thermanox coverslips. Cells were cultured in adipogenic media for 21 days, with media being changed every 3-5 days. After 21 days, cells were fixed in 10 % neutral buffered saline (NBS). Adipogenesis was confirmed using Oil red ‘O’ staining.

2.2.10 Chondrogenic differentiation:

Chondrogenic Media [modified from (Rust, 2004), (Kalia, 2007)]

High-glucose DMEM (4500 mg/L, D6429, Sigma-Aldrich, Dorset, UK)

1.0% P/S (0082, Invitrogen, Paisley, UK))

1.0×10^{-7} Dexamethasone, water-soluble (D2915, Sigma-Aldrich, Dorset, UK)

50 ng/mL Ascorbic Acid (A4544, Sigma-Aldrich, Dorset, UK)

1.0nM Sodium Pyruvate (P5280, Sigma-Aldrich, Dorset, UK)

10 ng/mL Recombinant Human TGF-β_3 (#100-36, PeproTech EC Ltd., London, UK)

5.0×10^5 BMSC were centrifuged at 2000 rpm for 5 minutes in universal containers. The pellets were left at the bottom of the universal containers and 10mL chondrogenic media was added. The lids of the containers were left loosely screwed on to permit gas exchange. The pellets were cultured in a standard humidified incubator, at 37°C with 5 % CO_2 (Nuaire DH Autoflow, Triple Red Laboratory Technologies, Long Crendon, Buckinghamshire, UK). After 24 hours, the pellets rounded up into small spheroid-shaped pellets. These pellets were cultured for 21 days, with media changes every 3-5 days, using supplemented chondrogenic media. After this time, the pellets were frozen in Tissue Tek® OCT compound (sakura Finetek, Zoeterwoude, Netherlands), and stored at -20°C. 10µm cryosections were made of the pellets, using a cryosectioning machine (Bright Instrument Company, Huntingdon, UK) at -20°C, and mounted onto 1.0-1.5 mm thick glass slides (BDH, UK) and stored at -20°C.

Chondrogenesis was investigated by Alician Blue and Sirius Red Histochemistry.

2.2.11 BMSC Passage Number

For osteogenic, adipogenic and chondrogenic differentiation, cells of passage four were used.

2.2.12 Histology-Osteogenesis

Von Kossa Assay

Von Kossa stain is used to detect mineral formation. As described above, cells were cultured on coverslips in osteogenic media. After 28 days, the culture media was discarded and the coverslips were washed with PBS and fixed in methanol for 10 minutes. The coverslips were then gently washed with distilled water three times and incubated in 1.5 % silver nitrate (762000, Hopkin & Williams, UK) for one hour. During this time they were placed under a source of light, which promotes the reduction of Ca^{2+} ions in the presence of silver nitrate. The coverslips were gently washed 3 times with distilled water after the silver nitrate was discarded. The coverslips were then covered with 2.5% thiosulphate (Fisions Scientific Apparatus, Loughborough, UK) for 5 minutes and gently washed with distilled water. Neutral Red (x634, Sigma-Aldrich, Dorset, UK), which stains nuclei red was used to counter stain the specimen for 5 minutes. Distilled water was used to rinse off excess stain until the coverslip was clear. Photos were taken using an Olympus BH2 photographic microscope and a JVC KY F55B Colour Video camera using zeiss Ks300 software (Imaging Associates, Thame, UK). Coverslips pre-treated with 5% acetic acid for 2 minutes acted as controls.

ALP quantitative biochemical assay (Cobas Bio)

A commercially available kit (AP307, Randox Laboratories, Antrim, UK) was used for quantitative analysis. The working reagent was prepared by mixing pre-weighed p-nitrophenol powder with 10 mL of diethanolamine buffer and heating the mixture to 37°C. Cell culture samples in media were washed in PBS and treated with autoclaved, distilled water at 37°C (a hypotonic solution) to lyse the cells. Samples were frozen and thawed three times and centrifuged at 10,000 rpm for 10 minutes. 50 µL of the resultant

supernatant was loaded into Cobas Bio® blue sample cups (AS diagnostics, Blackpool, UK). 0.25 mL of the p-nitrophenol phosphate working solution was added to each sample. The reagent and samples were loaded into the Cobas Bio® analyzer (Roche Lewes, UK) to run the assay. ALP activity measurements were expressed as U/L.

To compare results within groups, ALP activity readings were normalised for the number of cells by measuring the amount of DNA in each sample. This was done using the fluorometric dye Hoechst 33258, which binds specifically to the adenine-thymidine base pairs of DNA (Rago et al., 1990), (Rao and Otto, 1992). For the assay, lysed samples were transferred from cell culture dishes to Eppendorf tubes, which were spun in an ultracentrifuge at 10,000 rpm for 10 minutes, and the supernatant transferred to fresh tubes. A standard curve was prepared in a Fluoronunc® 96-well plate (Nunc, Rokskilde, Denmark), by diluting a stock, 1 mg/mL DNA standard (from calfthymus, D3664, Sigma-Aldrich, Dorset, UK) in saline sodium citrate (SSC) buffer, to 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 µg/mL, and loading 100 µL of the standards into the 96-well plate in triplicate. To prepare 500 mL of a 20x stock SSC buffer (20x the working concentration), 87.65 g of sodium chloride and 44.1 g of trisodium citrate was dissolved in distilled water and the pH was adjusted to 7.0 with 1M HCl or 1M NaOH.

A map of the 96-well plate was made to record the standard/sample order. 100 µL of each sample was loaded into the plate in duplicate. The Hoerchst 33258 dye (B2883, Sigma-Aldrich, Dorset, UK) was diluted from 1.0 mg/mL to 1.0 µg/mL in SSC and 100 µL added to each standard or sample well in the Fluoronunc® plate. Samples were run in a Fluoroskan Ascent® fluorimeter (Labsystems, Finland), using Genesis version 2.19 (Life Sciences UK Ltd.,) and Ascent Research Edition version 1.1.1 software

(Labsystems, Finland). The adenine-thymidine-Hoercht 33258-specific fluorescence was read at 460 nm. From the standard curve, the DNA concentrations could be calculated. For this, Microsoft Excel was used to plot the known concentration of the standards versus their absorbance at 460 nm, and a linear equation fitted to the resulting line. Equations having a correlation coefficient of 0.95 or above were acceptable for calculating the DNA concentrations of the samples.

ALP activity (U) per µg DNA was calculated as follows:

$$\text{ALP activity (U/L)} = \frac{\text{[ALP activity (U/mL)/100]}}{\text{Sample DNA concentration x 0.05 mL (volume of sample used for ALP assay)}}$$

2.2.13 Histology-Adipogenesis

Oil Red 'O' stain

A stock Oil Red 'O' solution was prepared by dissolving 0.5 g of oil red 'O' in 500 mL isopropanol and warming the bottle for 30-60 minutes at 56°C. A working solution was made by diluting 30 mL of the stock solution in 20 mL distilled water. After 14 days in adipogenic media, cells were fixed in neutral buffered formalin for 10 minutes, after which they were washed with distilled water and then stained in Oil Red 'O' for 10 minutes. After staining, cells were washed in distilled water and then counterstained using Harris' haematoxylin for 1 minute.

2.2.14 Histology- Chondrogenesis

Alician Blue and Sirius Red Histochemistry

To prepare the Alcian Blue stain, 1 g of Alcian Blue powder was dissolved in 100 mL of 3% acetic acid, and the solution filtered. The Sirius Red stain was prepared by dissolving 0.5 g of Sirius red F3B in 45 mL of distilled water. Absolute ethanol (50 mL) was then added, followed by 1.0 mL 1% sodium hydroxide. The solution was mixed vigorously, adding 4.0 mL of 20% sodium chloride, and the solution left to stand overnight before being filtered. Slide-mounted cryosectioned samples were fixed with methanol, after which they were washed in distilled water. They were then incubated with the Alcian Blue stain for 5 minutes, washed with distilled water, and then incubated in the Sirius Red stain for 1 hour.

2.3 Results

2.3.1 BMSC in Osteogenic media

Von Kossa assay-Visual Observation and Qualitative Analysis

After 28 days, those BMSC treated with osteogenic media had a greater positive reaction than those BMSC grown in normal media, with black deposits (Figure 2.1) indicating sites of mineralisation. Over 28 days, a multilayer of cells eventually formed three-dimensional folds, resulting in retraction of cells on the plastic surface and, thus, empty pockets of culture plastic became available for cell proliferation, similar to that noted by Schecroun and Delloye in 2003. There were no distinct changes in cell morphology over the 28 days. Negative control samples formed by low-passage BMSC treated with 5% acetic acid were negative for Von Kossa stain.

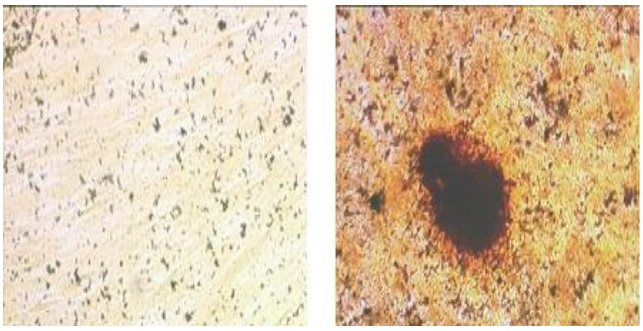


Figure 2.1 Von Kossa staining (10 x magnifications). Image on the left is low passage BMSC treated with 5% acetic acid whereas the image on the right is BMSC in osteogenic supplemented media. Note the calcium nodules staining black with Von Kossa stain

ALP Biochemical Assay

The levels of ALP (Figure 2.2) were significantly higher at days 7 ($p = 0.05$) and 21 ($p = 0.05$) when cultured in osteogenic media compared to standard supplemented media. On day 7, ALP activity in the BMSC grown in the osteogenic supplemented media was noted to be 12.61 ± 5.20 U/µg DNA, while the ALP activity in the cells grown in standard culture media was 2.35 ± 0.22 U/µg DNA. Similarly, the ALP activity was higher on day 21 in the osteogenic supplemented media (15.87 ± 8.97 U/µg DNA) compared to the standard media (1.52 ± 0.34 U/µg DNA). However, no significant difference was noted between the osteogenic supplemented media (8.31 ± 0.023 U/µg DNA and 1.26 ± 0.090 U/µg DNA) and standard media (7.92 ± 0.63 U/µg DNA and 1.68 ± 0.15 U/µg DNA) on days 14 and 28.

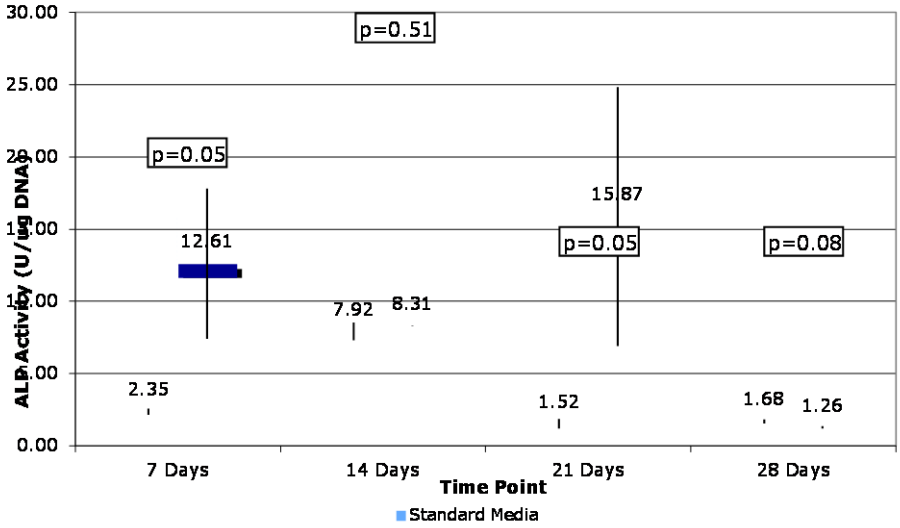


Figure 2.2 Graph illustrating ALP activity of cells at different time points. Maximum activity was seen at 14 days

2.3.2 Adipogenesis

Oil Red 'O' stain

Over the 14 days, cells changed morphology, becoming less spindle-shaped and shorter with long extensions. The oil red 'O' stain indicated the increased presence of lipids after BMSC were treated for 14 days adipogenic medium, when compared to those that were cultured in standard media. Although it must be noted that there were some cells staining positively for this lipid stain in the untreated BMSC cultures, it was to a much lesser extent than the adipogenic cultures

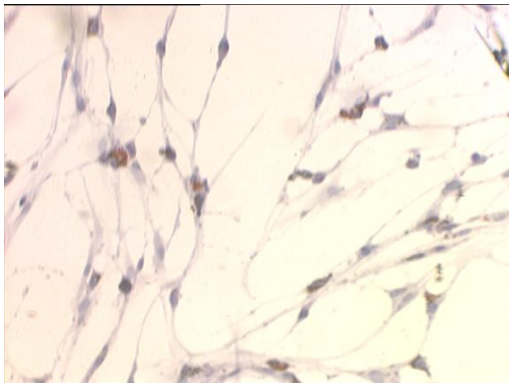


Figure 2.3 Red staining with Oil Red "O" stains confirming adipogenic differentiation of BMSC

2.3.3 Chondrogenesis

After 21 days in culture, all cell pellet cryosections showed a positive Sirius Red stain. However, only those samples treated with chondrogenic supplements including TGF-β3 showed a positive reaction with Alcian Blue (Figure 2.4d). Sirius Red is a non-specific collagen stain, while Alcian Blue is specific to GAGs. These results are in contrast to chondrogenic media excluding TGF-β3 and the control media containing only penicillin/streptomycin (Figure 2.4a).

Collagen I was found to be expressed in all cell pellets. Additionally, those cell pellets treated in complete chondrogenic media with TGF-β3 were found to express collagen II in small quantities. Although the collagen II stain had high background fluorescence, positive collagen II reactions could still be detected (Figure 2.4b, c-g).

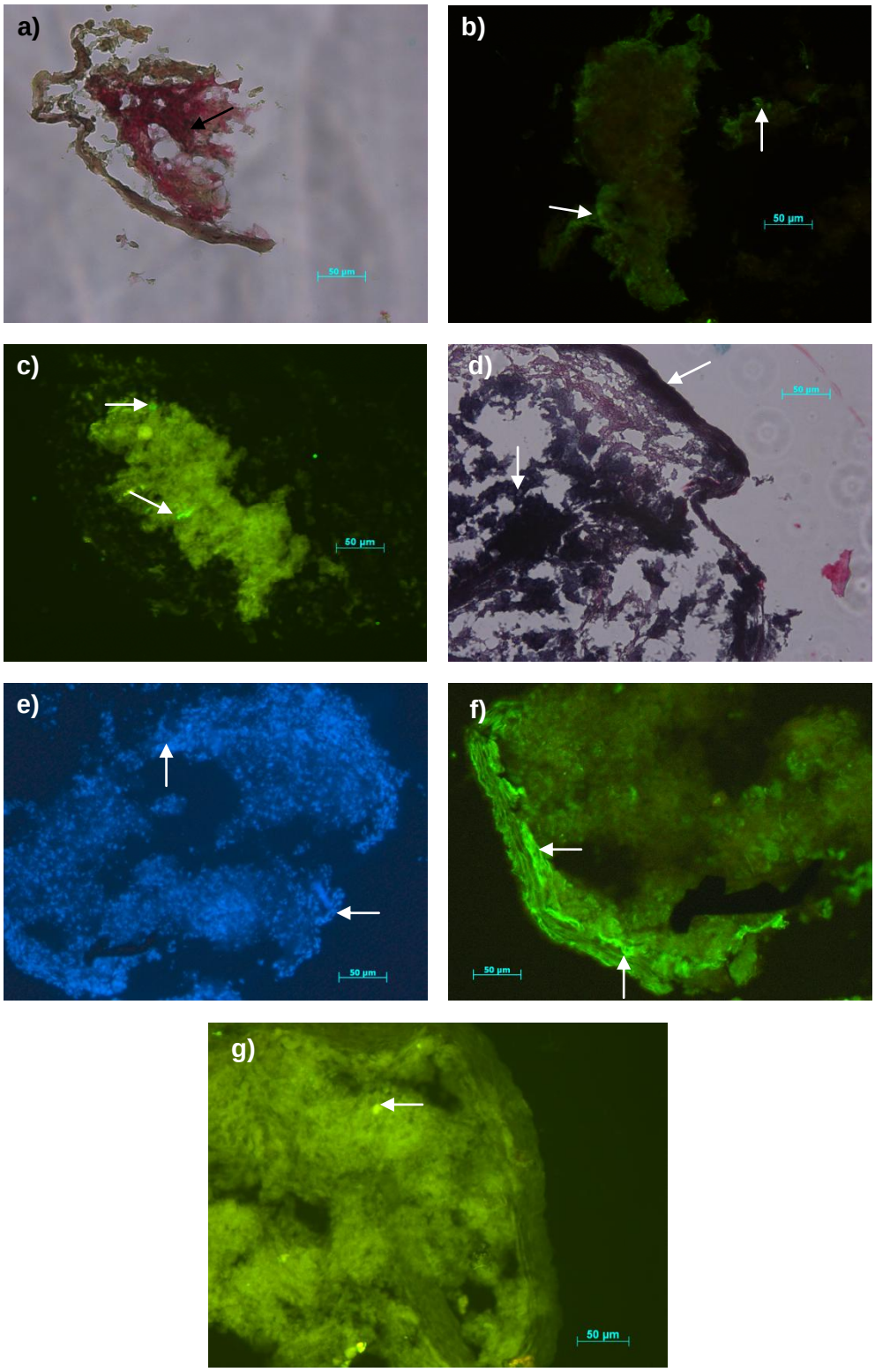


Figure 2.4 Stained cryosections of BMSC grown in DMEM+ (a-c) or chondrogenic medium for 21 days (d-g): a and d are sections stained with Sirius Red and Alcian Blue; b and f are stained for collagen I; c and g are stained for collagen II; e is a DAPI fluorescent nu

2.4 Spraying of BMSC

2.4.1 Methods and Materials

2.4.2 Suspension of BMSC in fibrin glue

As described above the BMSC were first isolated and expanded in culture until passage four. To suspend the cells in fibrin glue, they were trypsinised off tissue culture plastic after four passages and centrifuged to form a pellet. The pellet was resuspended in the thrombin component of fibrin glue at a concentration of 1.0×10^6 cells/mL.

The fibrin glue I used was Tisseel® fibrin glue (Baxter Health Care Ltd., Newbury, Berkshire, UK). This glue consisted of four components: a vapour-heated Tisseel® powder component (100-130 mg protein, of which approximately 75-115 mg is human fibrinogen); a bovine aprotinin solution; a vapour-heated human thrombin powder (45-55 mg protein containing about 500 IU^2 active bovine thrombin); a calcium chloride solution. The Tisseel® fibrinogen component was reconstituted in the aprotinin solution after a 10 minute incubation at 37°C in a device specifically created to heat fibrin components (Fibrinotherm®, Baxter Health Care Ltd., Newbury, Berkshire, UK). Ovine BMSC were put in a universal container and spun in a centrifuge at 2000 rpm for 5 minutes. The supernatant was then discarded and the pellet at the bottom of the universal container was resuspended in the 1.0 mL of reconstituted thrombin. Each component was then loaded into a 1.0 - 2.0 mL syringe (included in the Tisseel® package) and snapped into a dual-syringe holder with attached plunger device.

A 16-gauge cannula fixed to the end of the dual-syringe system was used to eject the BMSC-fibrin combination.

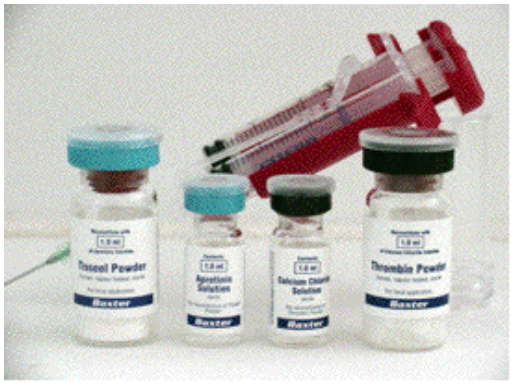


Figure 2.5 The dual syringe system used for spraying the BMSC suspended in fibrin glue is seen in the picture. Commercially available components of Tisseel® package is also shown.

2.4.3 Spraying BMSC in fibrin glue and fibrin-cell plug formation

To spray fibrin glue using this system, a Duploject® (Baxter Health Care Ltd., Newbury, Berckshire, UK) spray set was used, which consisted of sterile plastic tubing which connected to a dual-syringe tip, allowing air to come into contact with the fibrin components as they came out of the syringe. The other end of the tubing was connected to a Fibrijet® (Micromedics, Inc., St. Paul, Minnesota, USA) pressure gauge, in turn connected to a pressure pump. The Fibrijet was set to the desired pressure (1.0 Atm for the studies described in my thesis), and the plungers of the fibrin system pressed to allow release of sprayed fibrin onto the surface of the implants intra-operatively.

The effect of fibrin glue on BMSC viability, metabolism, proliferation, and morphology when applied using a spray or cannula has been studied previously (Kalia, 2007). The technique of spraying or the use of cannula does not affect the viability, metabolism or proliferation of BMSC.

The effect of fibrin glue on oBMSC viability, metabolism, proliferation, and morphology when applied using a spray or cannula

Viability

To assess the viability or death of cells after spraying, the Live/Dead assay was used. For this experiment, 2.0 x 10^6 cells/mL were suspended in thrombin and combined with fibrinogen, as described above, and 0.05 mL of each component was applied using a cannula, or with a pressurised spray, onto the tissue culture surface of Corning 6-well tissue culture plates. Cells were then incubated in media with 1.0 μM calcein and ethidium homodimer each (Invitrogen Ltd.), for one hour. After this time, samples were rinsed in PBS and viewed under a confocal microscope (Leica SP2 AOBS system, Leica Microsystems UK Ltd.), using Leica Confocal Software (LCS version 2.61, Leica Microsystems UK Ltd.) in 200 μm-thick sections, and were reconstructed in a 3D overlay using LCS Lite (version 2.61, Leica Microsystems UK Ltd.) software. Six samples were analysed per group. The four groups were: 1) oBMSC suspended in fibrin glue, applied with a cannula; 2) oBMSC suspended in fibrin glue, which was sprayed at 0.5 Atm; 3) oBMSC sprayed in fibrin glue at 1 Atm; and 4) oBMSC in fibrin glue, sprayed at 1.5 Atm. The percentage of live cells was calculated by dividing the number of live (calcein AM positive) cells by the total number of live and dead (ethidium homodimer-positive) cells. Results were compared to oBMSC initially plated at the same concentration, but on tissue culture plastic and without fibrin glue.

Proliferation

A thymidine-H^3 incorporation assay was used to determine the proliferation of oBMSC in fibrin glue when applied via a cannula or sprayed at different pressures. For this assay, 0.1 mL of fibrin & BMSC was cannulated onto the surface or sprayed at 1 Atm onto the well surface of 6-well plates (Orange Plastics, Triple Red Laboratory Technology), and covered with 2 mL of standard DMEM+. Proliferation was measured from 0-24 hours, 24-48 hours and 48-72 hours. For the 0 - 24 hours group, 1.0 μL of thymidine-H^3 (GE Healthcare) was added for each 1.0 mL of media, and placed in a standard humidified incubator for 24 hours. The assay was repeated in triplicate for each treatment and results were compared to oBMSC seeded at the same cell numbers as the cells suspended in fibrin, but cultured without fibrin glue.

After each 24-hour time point, cells were lysed with three repeated cycles of freeze-thawing. In order to break down the fibrin for analysis, samples were then freeze-dried overnight, resuspended in 0.5 mL of 1% papain (Sigma-Aldrich Co.) in PBS and incubated at room temperature overnight on a gentle shaker.

The Effect of Spraying BMSC in Fibrin Glue

Observation of Fibrin glue spraying at different pressures

Fibrin application at 1.0 and 1.5 Atm was visibly easier, smoother and more evenly distributed when compared to spraying fibrin at 0.5 Atm. At this pressure, the fibrin glue did not have sufficient pressure to properly form an aerosol.

Viability

There was no significant difference in the viability of cells after spraying at 0.5 Atm, 1.0 Atm, or 1.5 Atm in fibrin glue. Calcein was spread intracellularly, while the ethidium was visualised in the nucleus/nucleolar region of the cell. In all samples, some cells were seen to stain positively for both calcein AM and ethidium homodimer, suggesting plasma membrane damage that allowed ethidium penetration or cell death within minutes after spraying. The mean percentage of live cells in the groups sprayed at 1.5 Atm was 52.26 ± 5.08 %, while for the cells applied by a cannula, the mean percentage of live cells was 59.82 ± 13.36 % (Figure 8a). There was no significant difference between all groups ($p > 0.05$ in all comparisons).

Proliferation

Samples grown without fibrin glue (BMSC only) appeared to have less proliferation than those samples in fibrin glue applied with a cannula at 24 and 48 hours (35.95 ± 4.74 Bq/μg DNA/hour and 36.20 ± 11.06 Bq/μg DNA/hour in the oBMSC-only group; 79.33 ± 8.61 Bq/μg DNA/hour and 70.66 ± 7.73 Bq/μg DNA/hour in the cannula group). Cell proliferation was reduced after 24 hours in samples that had been sprayed, compared to cannula-applied controls (63.93 ± 6.00 Bq/μg DNA/hour at 24 hours, 6.61 ± 0.90 Bq/μg DNA/hour at 48 hours and 7.56 ± 1.81 Bq/μg DNA/hour at 72 hours; see Figure 8b). The difference in values were significant at 24 and 48 hours between the BMSC-only and cannula groups ($p = 0.01$, $p=0.03$ at 24 and 48 hours, respectively), at 24 hours between the BMSC-only and spray groups ($p=0.02$), and at 48 hours between the cannula and spray groups ($p=0.03$). Cell proliferation was reduced in all samples at 72 hours (19.32 ± 11.89 Bq/μg DNA/hour; 3.78 ± 0.66 Bq/μg DNA/hour; and 7.56 ± 1.81 Bq/μg DNA/hour, in the oBMSC-only (control, cannula and spray groups,

respectively). There was no significant difference between groups at this time point ($p > 0.05$ for all group comparisons).

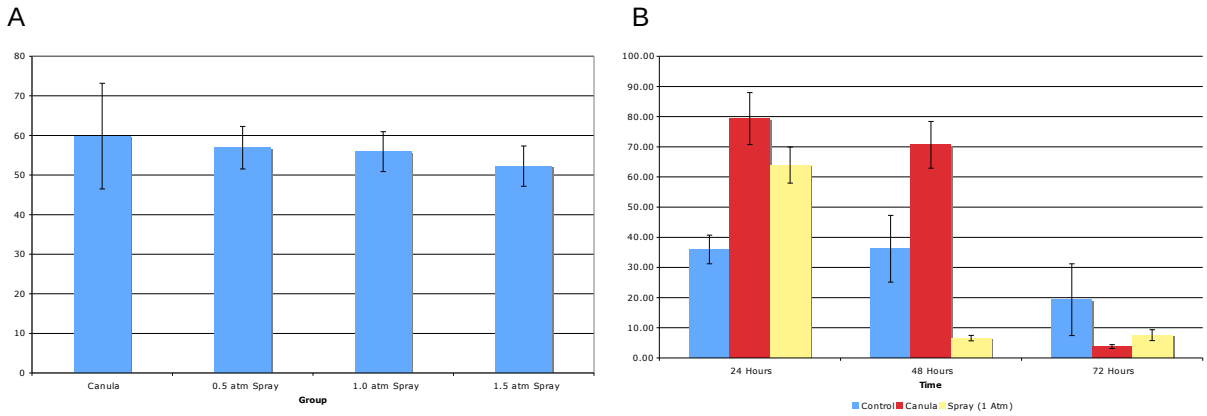


Figure 2.6 A) Chart indicating the percentage of live (Calcein AM positive) oBMSC in fibrin glue after being ejected from a cannula or using a spray, at 0.5 Atm, 1.0 Atm, and 1.5 Atm. B) Bar graph comparing 3H-thymidine incorporation (indicative of proliferation)

2.5 Discussion

This chapter confirms the ability of the cells isolated from the bone marrow aspirate to differentiate along osteogenic, adipogenic and chondrogenic lineages. The BMSC derived from the bone marrow were plastic-adherent cells that formed characteristic CFU-Fs and had a fibroblastic morphology.

For confirming the differentiation potential of the BMSC along osteogenic pathways, ALP assay and Von Kossa stains were used. Oreffo, et al. (1998) used the ALP assay to show osteoblastic differentiation of human BMSC. The ALP assay looks at ALP activity of a cell lysate but ALP on osteoblasts is expressed on the cell membrane. There exist a few different isoforms of ALP which are expressed in various tissues, such as the kidneys, liver and bone (Nakayama et al., 1998). Specific ALP antibodies have been used to show cell surface expression of ALP (Rust, 2004), (Gronthos et al., 1994),

although it has been suggested that the post-translational modifications to the carbohydrate side chains of the different ALP isoforms may not be easily detectable by commercially available antibodies (Nakayama et al., 1998).

The Von Kossa stain showed calcium deposition by the cells grown in osteogenic supplemented media. Similar results were noted in studies by Janssen et al. (2006) and Gorustovich et al. (2002).

The surfaces used to culture the cells described in this chapter as well as the surfaces used during differentiation, were slightly different in some cases. For example, Thermanox® coverslips were used to culture oBMSC in osteogenic media to show mineralisation after 28 days using the Von Kossa stain, whereas 6-well plates were used to culture cells for the ALP assay at 7, 14, 21 and 28 days. Each plastic surface is fabricated from different materials, and uses different techniques to treat the surface whether it is by using an electrostatic charge, or etching methods (EMS Catalog, 2007; (Scholz, 2003). This may, in turn, have resulted in varying conditions and reactivity to the differentiating supplements.

In future, other useful tests that could be used to characterise BMSC are a surface marker profile as well as to look for mRNA expression of genes involved in the different differentiation pathways, such as osteocalcin, osteopontin, ostrix, PPARy2, and collagen type II and X, and aggrecan (Lisignoli et al., 2006). One method that has been used to a lesser extent in the characterisation of BMSC is analysis of many different genes using a gene chip (Bourne et al., 2004); (Hishikawa et al., 2004).

It is unknown whether or not the anaesthetic regime used during bone marrow

aspiration affected the isolation, viability, or phenotype of the BMSC isolated from sheep. Halothane does not immediately exit the body, staying dissolved in fat and other tissues after the cessation of the anaesthetic regime. Also, the bone marrow was treated with 500IU of heparin per sample, in order to prevent clotting. The effects of heparin on BMSC or bone marrow cells has not been extensively reported in the literature, although it has been suggested that heparin-binding domains found in various elements of the extra-cellular matrix and the cell surface composition might affect cell signalling (Beauvais and Rapraeger, 2004); (Lopes et al., 2006). Also, the animals were under general anaesthesia with an inhaled anaesthetic (halothane) while bone marrow aspirates were taken. Bone marrow contains fatty tissue which can accumulate hydrophobic halothane molecules (Martin, 1994), which may in turn affect the BMSC. However, studies show that the first tissues to be exposed to halothane are those that receive a high fraction of cardiac output. The bone marrow is well vascularised, so a certain percentage of halothane probably did reach those cavities in the short term, even though the animals were not under anaesthetic for extended periods of time (time ranging from 10-45 minutes). Halothane has been shown to be carcinogenic *in vitro*, although one report did describe an increase in cancer cases in anaesthetists who had been repeatedly exposed to waste anaesthetic gas, as well as a rise in congentital abnormalities and spontaneous abortion (Martin, 1994). It is, therefore, possible that halothane could cause mutagenesis in the BMSC, or be accumulated in liposomes or any other lipid-containing structure in the cell for some length of time, before breaking down into its metabolic derivatives.

The experiments described in this paper show that BMSC survive spraying in fibrin glue at 1 atm. With regard to the spraying of cells in fibrin glue, it is unknown whether or not shear stress and fluid flow experienced by the cells in fibrin instigates an effect

on the cells differentiation or programmed cell death (apoptotic) pathways. Shear stress has been shown to induce osteocalcin expression in rat BMSC (Kreke and Goldstein, 2004) and may aid in the osteogenic differentiation of cells. In the Live/Dead experiment presented in this paper, some cells appeared to undergo cell death minutes after spraying, having first reacted with the "live" cell marker calcein AM to produce fluorescent calcein molecules. Whether or not this is apoptosis or necrosis due to irreversible cell damage could be determined by using apoptosis assays such as the terminal transferase dUTP nick end labelling (TUNEL) assay, which detects the DNA degradation step in apoptotic cells (Gavrieli et al., 1992), or protein detection methods such as immunohistochemical staining or western blotting for apoptotic markers such as Annexin V, Bax or p53 (Loewe et al., 2006), (Basu and Haldar, 1998). However, those oBMSC that do survive actively metabolise and proliferate, as shown using Alamar Blue and Thymidine-H^3 incorporation. Spraying did affect cell metabolism after the first 24 hours post-spraying, but cells appear to have recovered by Day 2 of the experiment. To look at the effects of spraying in fibrin glue on oBMSC osteogenic differentiation, one could perform RT-PCR detection of early osteoblastic genes such as cbfa-1 or osterix (Krishnan et al., 2003), (Pittenger et al., 1999).

Currie, et al. (2003) found no significant difference between spraying keratinocytes onto freshly debrided porcine wound sites in fibrin glue or without (cells suspended in culture medium) on the epithelial areas after three weeks *in vivo.*

In conclusion, this chapter summarises the isolation, expansion and characterization of BMSC along osteogenic, chondrogenic and adipogenic cell lineages. This confirms the multipotency of the isolated cells. Secondly, the technique of spraying the BMSC suspended in fibrin glue has no effect on the viability, metabolism or proloferation of

BMSC.

Thus, BMSC isolated from bone marrow aspirate may be differentiated along the osteogenic pathway by culturing them in osteogenic supplemented media and they can be suspended in fibrin and applied on to the surface of orthopedic implants by pressure controlled spraying techniques.

Chapter 3

3. Augmenting the Fixation of Uncemented Press-Fit Acetabular Cups in Total Hip Replacements Using Bone Marrow Stromal Cells (BMSC) Suspended in Fibrin Glue.

3.1 Introduction

This chapter investigates whether or not spraying autologous BMSC suspended in fibrin glue on the surface of HA-coated press-fit acetabular cups would increase bone formation and bone-implant osseointegration. A caprine hybrid THA model has been used for this study. Results of Chapter 2 confirm that BMSC sprayed on implant surfaces are viable and have the ability to proliferate and differentiate.

The hypothesis of this chapter is that:

Spraying autologous BMSC in fibrin glue onto the surface of HA-coated, press-fit acetabular components would increase bone formation around the implant, improve bone-implant contact, and reduce fibrous tissue at the bone-implant interference compared to fibrin glue-treated controls in a caprine model.

The aims and objectives of this chapter

1) To spray BMSC at a concentration of 10 x 10^6 cells in fibrin glue onto the outer surface of HA-coated press-fit acetabular cups of THAs in an *in vivo* caprine model;
2) To use force plate analysis to differentiate loading of operated and non-operated limbs and to compare between the study and control group;
3) To quantify the new bone formation at the implant bone interface using histological techniques and compare this in the study and control group;
4) To quantify the bone-implant contact along the interface by histological techniques and compare this in the study and control groups.

3.2 Materials and Methods

3.2.1 Animal model selection

Sannan goats were selected for this study. These animals were skeletally mature (two to five years of age), and weighed between 65-90 kg. All procedures were carried out according to the Home Office Animals Scientific Procedures Act of 1986.

3.2.2 Procedure for BMSC isolation and characterisation

Animals were cared for as described in Chapter 2. BMSC were isolated, culture expanded and characterised as described in Chapter 2. The BMSC were differentiated along osteogenic, chondrogenic and adipogenic pathways to confirm multipotency.

3.2.3 Suspension of BMSC in fibrin glue and the technique of spraying BMSC on orthopaedic implants

The suspension of BMSC in fibrin glue and the technique of spraying BMSC on the surface of implants are detailed in Chapter 2. BMSC were prepared as described in Chapter 2. On the day of the surgical procedure, 10×10^6 BMSC were suspended in 1.0 mL of reconstituted thrombin.

10×10^6 cBMSC were suspended in reconstituted thrombin (Tisseel®, Baxter Health Care Ltd.). The Tisseel® components were assembled in the operating theatre per-operatively.

The technique of spraying BMSC and suspension in fibrin does not affect the viability, metabolism or proliferation of BMSC (Chapter 2 section 2.4.3).

3.2.4 Experimental groups

Two groups were studied. Twelve goats underwent total hip replacements and were randomly assigned to the two groups.

Control Group: Total hip replacement with cemented femoral components and press-fitted, HA-coated acetabular components sprayed with fibrin glue (WITHOUT CELLS);

Experimental group: Total hip replacement with cemented femoral components and press-fitted, HA-coated acetabular components treated with 10 x 10^6 BMSC in a fibrin glue spray.

3.2.5 Implant Design & manufacture

Custom made goat implants (total hip replacements) were manufactured for this experiment. Titanium alloy (TiV_4Al_6) was used to manufacture the femoral component for cemented fixation and acetabular cup for uncemented fixation was used (Figure 3.1). The acetabular cups were coated with a layer of HA approximately 50μm in thickness using a plasma spray technique (Plasma Biotal Ltd. Tideswell, North Derbyshire, UK). Each cup had eight cutting flutes equally spaced around the periphery of the cup to provide rotational stability after fixation. The femoral head was made of cobalt chromium and polyethylene acetabular cup liners were used.

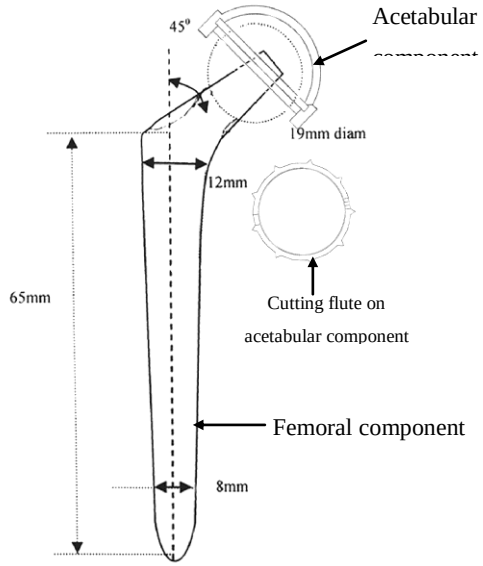


Figure 3.1 Diagram of total hip replacement used in caprine model, which included a cemented femoral component and an uncemented acetabular cup with an ultra high molecular weight polyethylene liner.

3.2.6 Surgical Procedure

All animals were handled and cared for as described in Chapter 2 (Materials & Methods). The pre-operative preparation and anaethesia are as described in Chapter 2 also. For total hip replacement, right hips were routinely used in all case. The region over the greater trochanter was prepared for surgery using povidine-iodine solutions as previously describe in section 2. The animals were placed on their left side on the operating tables and a posterior approach was used to access the hip joint. Once dislocation had been achieved, a femoral neck osteotomy was performed, and the femoral head excised. The acetabulum was then prepared using reamers of increasingly larger sizes until the desired equatorial diameter was achieved, and bleeding subchondral bone was uncovered.

In the experimental group animals, 2 ml of autologous BMSC in fibrin glue was sprayed

onto the HA-coated surface of the acetabular cups at 1.0 atm of pressure. To do this, the fibrinogen and thrombin components of the fibrin glue were transported to the operating theatre with or without BMSC (see Chapter 2) and maintained at 37° C in a Baxter Fibrinotherm® device (Baxter Health Care Ltd., Newbury, Berkshire, UK) until used. Each component was loaded into a 1.0 mL syringe (included in the Tisseel package) and snapped into a dual-syringe holder with attached plunger device.

To spray the fibrin glue using this system, a Duploject® (Baxter Health Care Ltd., Newbury, Berkshire, UK) spray set was used, which consisted of sterile plastic tubing which connected to a dual-syringe tip, allowing air to come into contact with the fibrin components as they came out of the syringe. The other end of the tubing was connected to a Fibrijet® (Micromedics, Inc., St. Paul, Minnesota, USA) pressure gauge, which was connected to a pressure pump (see Chapter 2). The Fibrijet was set to 1 atm and the plungers of the fibrin system allow release of aerolised fibrin onto the surface of the HA-coated implant. The implants were slowly turned as the fibrin was applied, to allow an even distribution around the surface of the cup collar (figure 3.2).

For implants in the study group, the acetabular shell was coated in a layer of 2 ml fibrin glue containing 10 x 10^6 BMSC (Figure 3.2). After three minutes, once the fibrin was firmly set, the acetabular component was impacted into position. At this point, some fibrin glue would be squeezed out around the rim of the cup and was observable by eye.

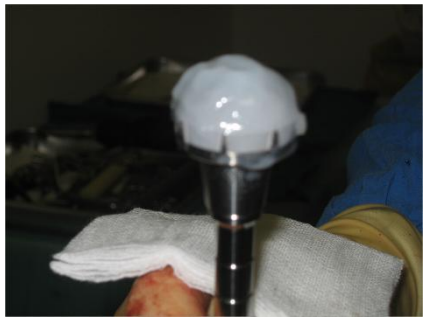


Figure 3.2 Photograph of a caprine acetabular cup which has been coated with a fibrin glue spray.

In the control group only fibrin glue was sprayed before the cup was impacted into position.

The femur was then prepared by first removing trabecular bone with a curette, and repeatedly washing with normal saline solution (0.9% sodium chloride solution). A cement restrictor was inserted in the femoral canal. Bone cement (Refobacin® Bone Cement, Biomet Europe) was then applied and pressurised into the canal, prior to insertion of the femoral component. Once the cement had set, the hip was reduced, and the tissue sutured.

A spray bandage (Opsite®, Smith & Nephew, Gallows Hill, Warwick, UK) was applied to the wound. Antibiotic and analgesic prophylaxis was administered daily with subcutaneous injections of Exenel™ (ceftiofur hydrochloride, 1 ml/50 kg, Pfizer Inc.) and IM injections of Vetergesic™ (buprenorpine, 0.6 mg/animal, Reckitt and Colman Products Ltd.,) for three days post-operatively.

3.2.7 Post -operative radiographs

Post-operative radiographs were routinely taken to assess the position of the components and to look for the presence of any intra-operative fractures (see Figure 3.3).

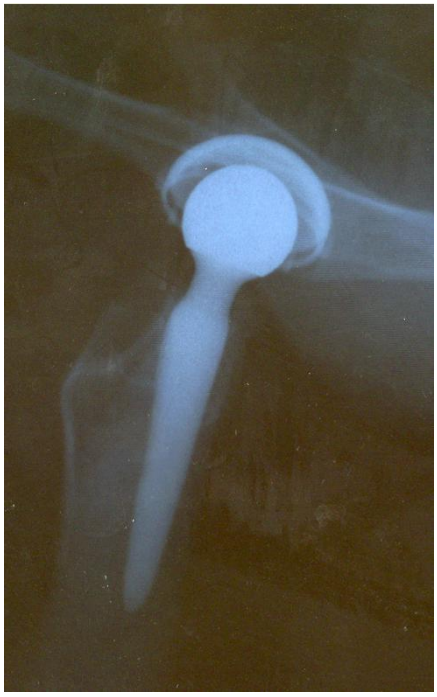


Figure 3.3 Radiograph showing caprine THA in situ post-operatively

3.2.8 Measurement of Ground Reaction Force

To compare the function of the operated limb between groups, the ground reaction force was measured at 3, 6, and 12 weeks post-operatively. Animals walked over a force plate and the force transmitted through the operated (right) and unoperated (left) limb was recorded. The maximum force was recorded (F_{max}) and expressed as a percentage of force through the right limb compared to the left limb, or $F_{right\ max}/F_{left\ max}$.

Animals were sacrificed with an overdose of 20% pentabarbitone (0.7 mg/kg, J.M. Loveridge Ltd. Southampton, UK).

3.2.9 Undecalcified Hard-Resin Histology

Following sacrifice, all right acetabulae were prepared for histology by stripping excess soft tissue and fixing in 10% neutral buffered formalin (NBF) for a minimum period of

one week. Specimens were dehydrated in increasing concentrations of industrial methylated spirit (IMS). Samples were de-fatted in chloroform and immersed in a 50% IMS, 50% resin mixture (LR White Resin, London Resin Company Ltd., Reading, Berkshire, UK), followed by immersion in 100% resin. Samples were then cast using a polymerisation accelerator (LR White Accelerator, London Resin Company Ltd., Reading, Berkshire, UK). Specimen blocks were sectioned at four sites, using a water-cooled band saw with a diamond-edged blade (Exact saw, Germany). Thin sections (approximately 80 µm thick) were prepared (Exact grinding machine, Germany; and Exact Polishing machine, Germany) and stained with Toludine Blue (Bancroft and Stevens, 1986) and Paragon (Tanzer et al., 2003) to stain soft tissue and bone, respectively. Stained slides were then viewed under an Olympus light microscope and images digitally captured using a JVC KY F55B Colour Video camera. Bone area and bone-implant contact was quantified using image analysis software (Zeiss KS300 software, Imaging Associates, Thames, UK).

3.2.10 Bone Area

New bone area adjacent to the cup was measured using a line-intersection method, where four lines radiating from the centre of the cup were used to divide the cup area into five zones (see Figure 3.4a). New bone area was quantified outwards from the cup within 1 mm^2 of the surface within the 5 zones, and was quantified in units of mm^2.

3.2.11 Bone-Implant contact

A line intercept method was employed to measure the bone implant contact where the presence of bone at the interface was measured at the intersection of 34 lines radiating

from the centre of the radius of the cup (Figure 3.4b). Bone implant contact was expressed as a percentage.

3.2.12 Fibrous Tissue measurement

Where present, the thickness of the fibrous tissue from the metal backing to the bone surface was measured along the radiating line.

For the purpose of analysis, the 5 zones around the cup and 35 regions were also grouped into two categories: the periphery and the dome of the cup.

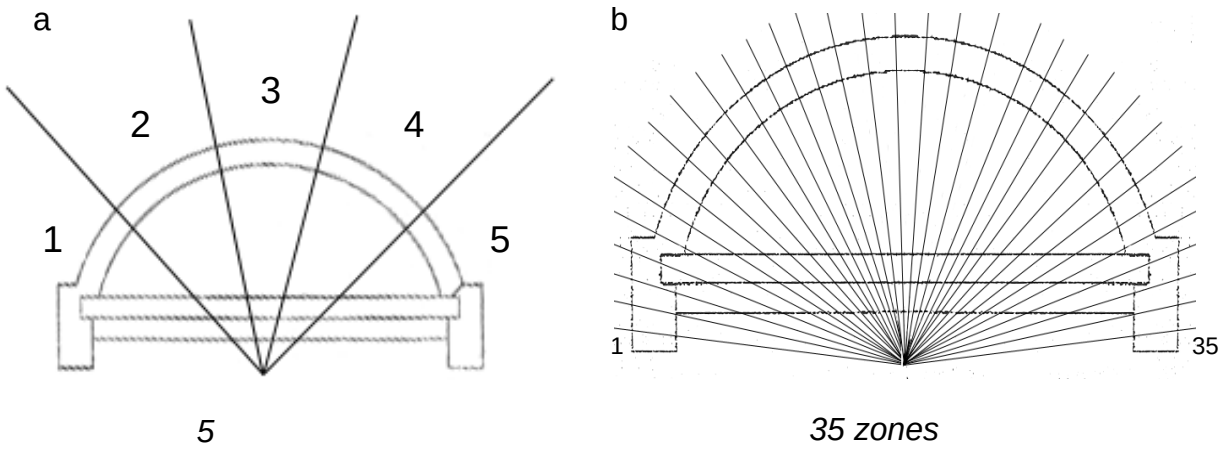


Figure 3.4 a) Diagram of acetabular cup divided into 5 zones for quantification of new bone formation at the bone-implant interface; b) Diagram of acetabular cup divided into 35 zones for analysis of bone-implant contact and fibrous tissue thickness.

3.2.13 Statistics

To test the normality of the distribution of the data, the Kolmogorov-Smirnov test was used, using SPSS 11.0 for Mac OSX. Parametric data was analysed using an independent-samples T test; non-parametric data was analysed using the Mann-Whitney *U* test. Results were considered significant when $p < 0.05$.

3.3 Results

3.3.1 Post-operative Results

Animals were allowed immediate mobilisation and all were fully weight-bearing within 48 hours. All twelve animals were weight-bearing and healthy for the 12-week period.

3.3.2 Measurement of Ground Reaction Force

Pre-operatively, goats were tested for their Ground Reaction Force at 6 and 12 weeks. When data was expressed as the percentage loading of the operated (right) leg compared to the un-operated (left) leg, there was no significant difference in loading between groups at both 6 weeks (Controls-79.74 ± 3.63%, BMSC-59.39 ± 9.33%, p=0.086) and 12 weeks (Controls-86.0% ± 2.85%, BMSC-62.33 ± 5.12%, p=0.055). Figure 3.5 displays these results.

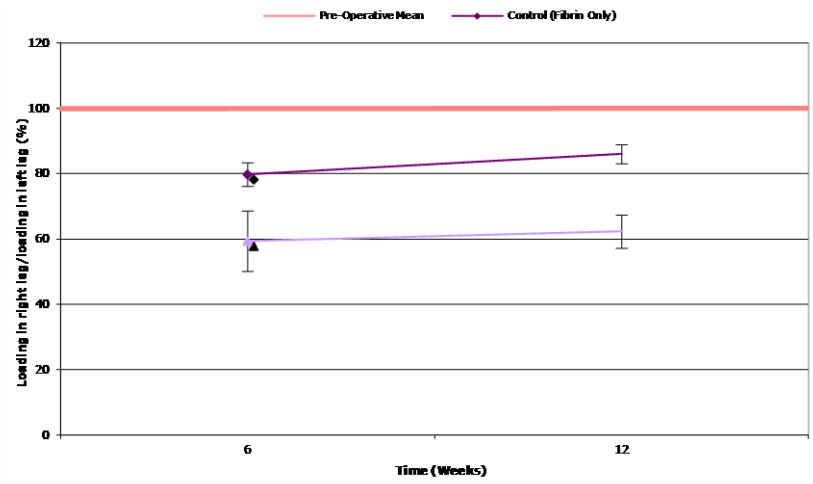


Figure 3.5 Graph displaying force plate analysis results at 6 and 12 weeks. Results were normalised to pre-operative loading values (100%).

3.3.3 Macroscopic analysis at retrieval

Upon retrieval of the acetabular components, four of the six acetabular cups sprayed with BMSC group had a noticeable cuff of bone formation over the rim of the cups. None of the cups with sprayed cells were loose. Out of the six implants in the control group, two of the acetabular cups appeared loose (that is, were not firmly attached to the acetabulum by tissue).

3.3.4 Histology

In BMSC-group sections, a thick layer of bone in contact with the acetabular cup surface could be seen, with little fibrous tissue present (Figure 3.6b). This was in contrast to the control group, which had a fibrous tissue layer interposing between the bone and implant surface, and as a result, less bone-implant contact (Figure 3.6a).

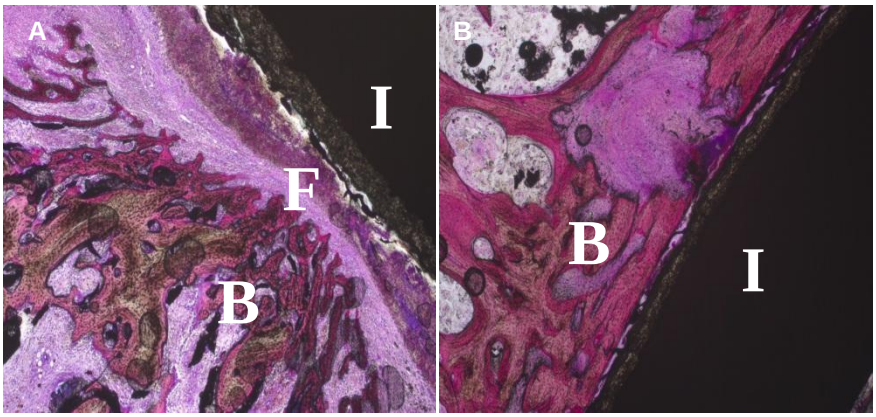


Figure 3.6 Histological sections showing the acetabular cup-bone interface in the A) control group, and B) BMSC-treated group.

New Bone Formation

New bone formation in Zone 5 showed a significant increase in the BMSC group (71.97±10.91%), when compared to the controls (23.85±15.13%, p=0.028, Figure 3.7). The other zones did not show a significant difference in new bone around the cup.

Overall, new bone growth in the BMSC group was 30% greater than the control group (71.42±8.97% and 54.22±16.56%, respectively, p=0.58).

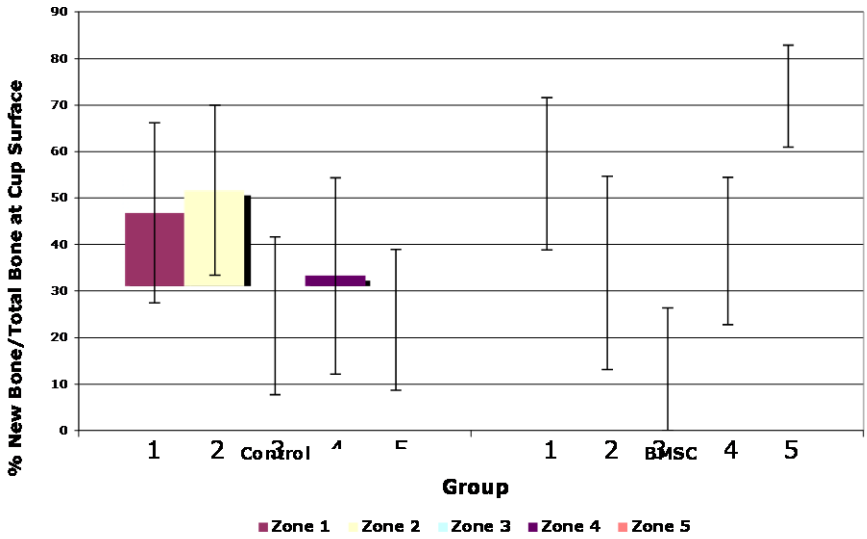


Figure 3.7 Bar graph displaying new bone formation (%) in each of the 5 zones around acetabular cups in control and BMSC-treated groups.

Bone-Implant Contact

Overall, bone-implant contact was significantly increased in the BMSC group (30.71 ± 2.95%), in contrast to the control group (5.14 ± 1.67%, p=0.014). When this was divided into two regions, the periphery and dome of the cup, it was found that bone-implant contact was significantly increased in BMSC-treated cups around the periphery of the cups (34.29 ± 11.61%), when compared to controls (7.14 ± 2.61%, p=0.01, Figure 3.8). However, there was no significant difference in bone-implant contact at the dome of the cup between the two groups (19.84 ± 16.19% in the control group, 20.95 ± 5.75% in the BMSC-treated group, p=0.165).

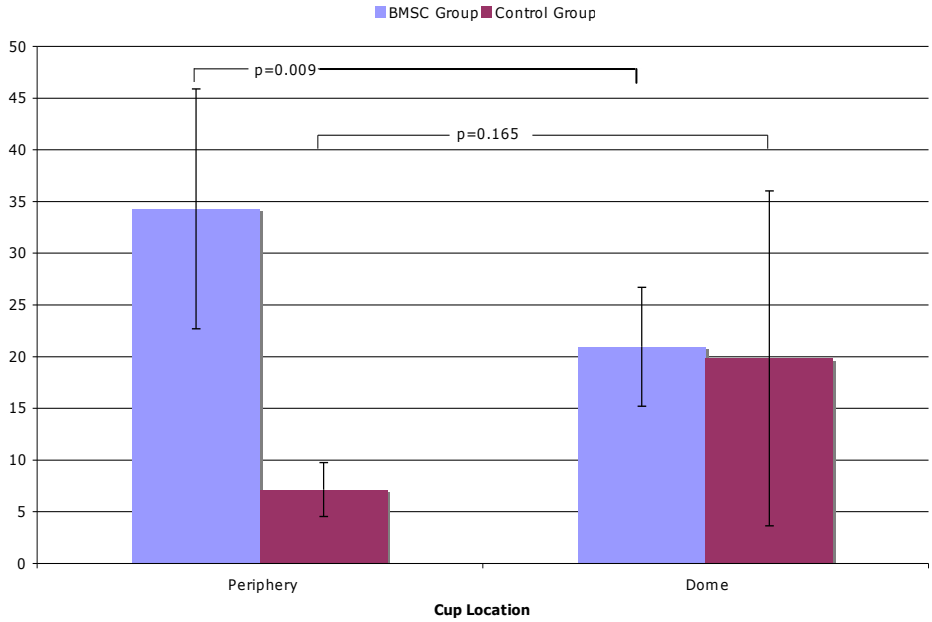


Figure 3.8 Graph showing bone-implant contact around the periphery and dome of cups in both experimental groups.

3.3.5 Fibrous Tissue Analysis

Fibrous tissue thickness at the periphery of the cups was significantly reduced in BMSC-treated group cups (327.49±20.38 mm), in contrast to control group cups (887.21 ± 158.89 mm, p=0.02, Figure 3.9a). There was no significant difference in fibrous tissue thickness found at the dome of the cup (902.45 ± 80.67 mm in the control group and 739.1 ± 173.72 mm in the BMSC group, p=0.47, see Figure 3.9b).

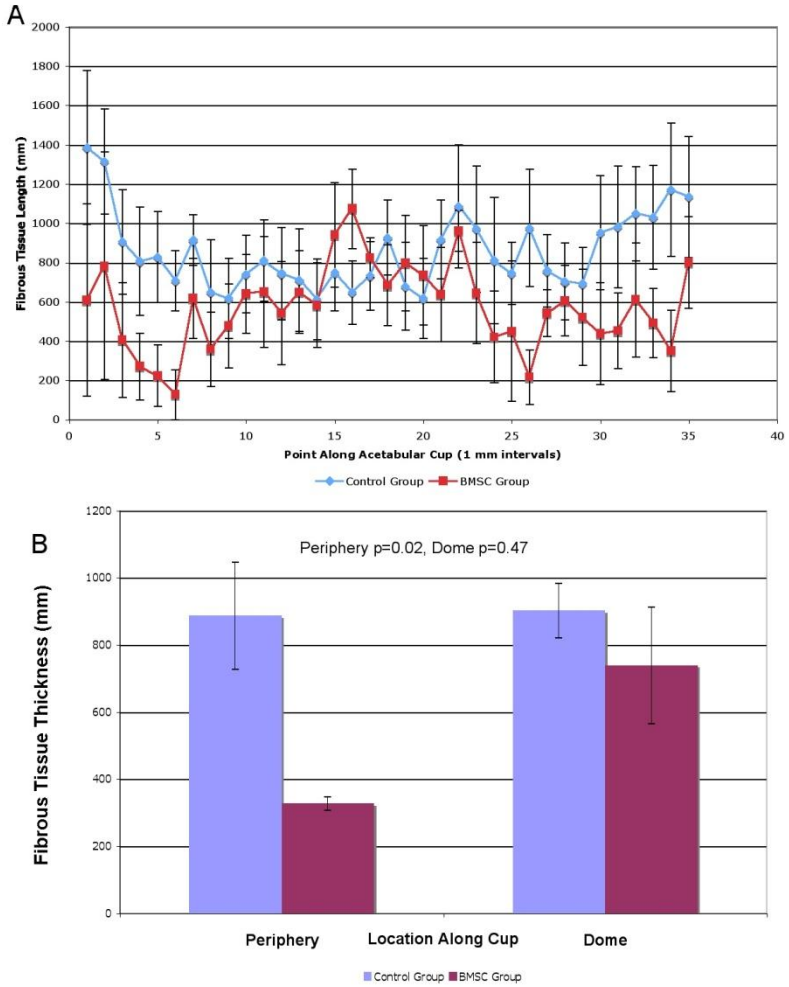


Figure 3.9 A) Plot of fibrous tissue length across all 35 acetabular zones comparing control and BMSC-treated groups. B) Mean fibrous tissue thickness around the acetabular

3.4 Discussion

BMSC suspended in fibrin glue and sprayed on the surface of HA-coated acetabular cups increase bone implant osseointegration and bone implant contact and decrease fibrous tissue at the bone implant interface.

Hydroxyapatite (HA) coatings have been used on orthopaedic implants to promote fixation in the host bone (Blunn et al., 2000), (Coathup et al., 2005), (D'Antonio et al., 1992); (Geesink, 1993). Clinically, HA coatings are used extensively on femoral and

acetabular THA components (Froimson et al., 2007), (Overgaard et al., 1999), (Capello et al., 2006). HA coatings have also been applied to uncemented massive bone tumour prosthesis, where they have improved implant fixation and contributed to enhanced implant survival (Unwin, 2005). In large animal models, these coatings have been shown to improve osseointegration of the implant to host bone and prevent wear particle migration (Coathup et al., 2005), (Geesink et al., 1988). Geesink, et al. (1988) showed that in a canine model that femoral components with HA coating and left *in situ* for 10.6 years, there was no evidence of wear debris-associated osteolysis. A study by Coathup, et al. (2005) comparing different surfaces and their effects on bone-implant contact and the prevention of wear-particle migration in an accelerated wear ovine model found that a porous, HA-coated surface was most effective.

HA is osteoconductive (Geesink et al., 1988), and has been shown to promote the accumulation of bone morphogenetic proteins (Geesink, 2002). To promote osteoinductivity, HA has been combined with bone morphogenetic protein 2 (BMP-2) and has been proven to improve bone growth and implant fixation in animal models (Sachse et al., 2005), (Aebli et al., 2005). It may also be possible to promote early fixation and increase bone implant contact around implants such as the acetabular component of total hip replacements by treating the implant surface with osteoblast precursor cells such as bone marrow stromal cells (BMSC). This may prevent the migration of wear particles to the bone-implant interface and reduce longer-term aseptic loosening. This chapter describes a BMSC-fibrin spray technique, developed in order to apply cells to the implant surface and the effect of using this on acetabular implant fixation in a large animal model.

With regard to the *in vivo* experiment reported in this paper, there was no significant difference in loading of the operated legs between groups, indicating that function has

not been compromised by the addition of BMSC, and that the results are not associated with better recovery or increased loading of one group compared to the other.

A radiographic evaluation would be useful in future studies, as well as a longer-term investigation looking at the effectiveness and survival of BMSC over a greater period of time. It has previously been reported (Coathup et al., 2005) that improved fixation reduced osteolysis at the bone-implant interface, and that the fixation was greatest when a porous HA coating was used on the uncemented acetabular cup. The HA-coated, grit-blasted group that was included in the study did not perform as well as the porous HA-coated cups, and was, therefore, selected for this study as being a less-than-perfect situation where improved cup fixation would be beneficial.

It may also be beneficial to apply the BMSC-fibrin glue system to HA-coated, uncemented femoral stems and in other situations where osseointegration of implants is important. A previous study by Kalia, et al. (2006) showed that when autologous, ovine BMSC were sprayed in fibrin glue over the HA-coated collars of mid-shaft tibial replacements used as a model of fixation of massive segmental bone tumour implants, the bone integration of the collar was improved when compared to controls.

Bone formation was significantly increased around the periphery of the cup in the BMSC-treated group, when compared to controls, whereas, at the dome of the cup, there was no significant difference. A variation in cell distribution around the acetabular cups in the BMSC-treated group may account for this result, as fibrin was pushed out from the surface of the acetabular component towards the implant periphery. Subsequently, as the fibrin was combined with the BMSC, the cell numbers may have been higher at the acetabular cup periphery than at the dome.

Additionally, a variation in mechanical forces applied to the BMSC around the acetabular component may have influenced bone formation. Studies looking into the sensitivity of BMSC to mechanical forces (Nagatomi et al., 2003), (Hamilton et al., 2004), (Ignatius et al., 2005) have shown that an application of compressive forces can cause osteoblastic differentiation of BMSC. Ignatius, et al. (2005) demonstrated increasing expression of the master osteoblastic gene CBFA-1 after mechanical stretching of a human osteoblast precursor cell line (hFOB 1.19). It has been proposed that this mechanical effect is regulated by the MAP kinase (MAPK) signalling pathway, using extracellular signal-regulated kinase 1/2 (ERK 1/2) (Simmons et al., 2004). When the press-fit cup was impacted into place in the goat, it is possible that the deformation of the cup and fit of the cup in the acetabula caused increased compressive forces closer to the periphery of the cup, inducing enhanced osteoblastic differentiation by the BMSC. Cutting flutes at the edges of the cup may have also altered the loading situation. It would be useful to measure the forces applied by the cup on the surrounding bone using telemetry, as has been done to measure joint contact forces (Graichen et al., 1999), to see if this correlated with the pattern of increased bone formation around BMSC-treated cups. It may also be possible that the fibrin glue, once impacted into the acetabulum, may have migrated towards the periphery of the cup, along with the enmeshed cells, resulting in greater bone formation at this location simply because there was more fibrin present. This movement of the fibrin may be useful in improving the filling of gaps between the implant and bone, which is a property attributed to hydroxyapatite (Geesink et al., 1988), (Overgaard et al., 1999). This property may be important for filling in defects around revision implants. Also, increasing bone formation and decreasing fibrous tissue around the periphery of the cup could be effective in preventing the migration of wear particles between the bone-implant interface and the resulting osteolysis. Fibrous tissue is known to be a conduit of wear

particles, and previous work has shown that sealing the implant from wear particles using a non-resorbable, ePTFE membrane (Bhumbra et al., 2000) in a goat THA wear-particle producing model was effective in preventing particle migration.

In future, it would be useful to be able to track the implanted cells' contribution to new bone formation around the implants, by using a marker such as GFP or LacZ (Zhou et al., 2005). It may be possible that the BMSC in this study promote bone growth in two ways, by direct differentiation into bone-producing osteoblasts, or by the release of cytokines that promote the homing of host cells to the site, and augment the bone healing response (Caplan and Dennis, 2006).

3.5 Conclusions

Spraying BMSC in fibrin glue onto HA-coated, press-fit acetabular cups significantly increased bone area around the implants, improved bone-implant contact, and reduced the amount of fibrous tissue around the cup. This study may have clinical implications as this technique may increase the early fixation and reduce osteolysis around acetabular components of primary THAs or hip resurfacing procedures, thereby, increase longevity of the implants. BMSC may also have a beneficial role in regenerating bone if applied to revision situations where poor bone stock is an issue.

Chapter 4

4. The Effect Of Guided Bone Regeneration And Varying BMSC Dosages On Osseointegration Of HA Coated Implants

4.1 Introduction

The previous chapter has demonstrated the ability of BMSC to produce new bone and improve bone-implant contact in a large animal model. However, there may be an optimum BMSC dose required to stimulate maximum bone formation *in vivo*. It may be possible to stimulate the BMSC in an osteogenic supplemented media to improve bone formation. Guided bone regeneration techniques when combined with the cell therapeutic treatment investigated in my thesis may further enhance bone formation.

Expanded-polytetrofluoroethylene (e-PTFE, Gore-Tex™) facilitates new bone formation by sealing the defect site from the surrounding tissue (Bhumbra et al., 1998). E-PTFE is a biocompatible, non-resorbable, synthetic membrane that has been used in guided bone regeneration. The membrane used in my study had three layers. Its inner layer, which has 1 micrometer pores, prevents the in-growth of cellular material such as fibrous tissue, whereas water and various macromolecules and growth factors can pass through freely. The outer layers are more porous and allow for tissue integration. This membrane can be used to increase bone regeneration into defects and the technique allows bone to grow directly around an implant (Bhumbra et al., 1998). E-PTFE has also been shown in a large animal model to effectively prevent wear-particle induced osteolysis at the acetabular cup implant-bone interface created by total hip replacements, significantly reducing the aseptic loosening of these implants (Bhumbra et al., 2000). The use of e-PTFE with various cell concentrations of BMSC in this study may prevent cell migration from their original site of implantation.

The hypotheses of this chapter are:

1) **There is an optimum cell concentration at which BMSC produce maximum new bone.**

2) **Stimulating BMSC in osteogenic media should increase the quantity of new bone formation.**

3) **Guided bone regeneration is possible using e-PTFE and BMSC.**

The aims and objectives of this chapter are:

1) To investigate the effects of autologous BMSC in varying cell dosages (1.0×10^5 and 10×10^6 BMSC/mL) as well as fibrin alone on the amount of new bone formation in a caprine trans-cortical model, within a 1.5 mm gap defect in a in vivo model.

2) To investigate the effect of autologous BMSC treated in osteogenic supplemented media (10×10^6 cells treated in osteogenic supplemented media) on the amount of new bone formation in the same model. The BMSC treated in osteogenic supplemented media were tested for osteoblastic differentiation as described in Chapter 2 (section 2.2.12).

3) To investigate the effect of using e-PTFE with the autologous BMSC on the amount of new bone formation in the same model.

4) To prepare histological sections and measure the amount of new bone formation adjacent to the implants and within the gaps.

4.2 Materials and Methods

Sannan goats were selected for use in this study. These animals were skeletally mature (two to five years of age), and weighed between 65-90 kg. All procedures were carried out according to the Home Office Animals Scientific Procedures Act 1986.

4.2.1 Isolation and Expansion of autologous BMSC

Caprine BMSC were isolated as previously described (See Chapter 2, Materials and Methods).Cells were expanded to passages 3 and 4 for use in this study. The BMSC were differentiated in osteogenic supplemented media (as described in Chapter 2, section 2.2.8 and the cells were tested for osteogenic potential (as described in Chapter 2, section 2.2.12).

4.2.2 Implant design and manufacture

The implants were small partially-threaded transcortical screws similar to that used by Bhumbra, et al. (1998) and (Kalia, 2007). The implants were manufactured from titanium alloy (T.6A14V). The screws were 25 mm in length, and the distal M5 threaded region was 5.0 mm in diameter. A cutting flute was located at the bottom for ease of insertion into the cortical bone. The top half of the screw was 3.0 mm in

diameter and was plasma sprayed with a highly crystalline, thin (<50 micrometer thickness) HA-coating (Plasma Biotal, Tideswell, North Derbyshire, UK).

4.2.3 Treatment groups

Four implants were inserted into the left and right tibia of each sheep, with a total of eight implant groups per sheep. The centres of the drilled holes were 3 cm apart in all cases. An e-PTFE covering the implant and defect at the near cortex was used on the right leg, while no e-PTFE was used on the left leg. Butyl-cyanoacrylate glue (Vetbond™, National Veterinary Supplies. Talke Pits, Stoke-on-Trent, UK) was used to adhere the e-PTFE membrane to the bone.

Table 4.1 outlines the treatment of the eight groups investigated, which were rotated clockwise in each successive animal to enable site dependent effects of the treatment to be evaluated. Cells were administered as described in Chapter 1 via incorporation within fibrin glue and sprayed onto the surface of the unthreaded, HA-coated segment (3mm diameter half of the 25 mm screw, see section 4.2.2) of the implant at 1.0 atmosphere of pressure.

Left tibia (no-PTFE)	**Right tibia (PTFE)**
Fibrin only	Fibrin only
1.0 X 10^5 auto BMSC/ml in fibrin	1.0 X 10^5 auto BMSC/ml in fibrin
10 X 10^6 auto BMSC/ml in fibrin	10 X 10^6 auto BMSC/ml in fibrin
10 X 10^6 auto BMSC/ml (in osteogenic media) in fibrin (ogBMSC)	10 X 10^6 auto BMSC/ml (in osteogenic media) in fibrin (og BMSC)

Table 4.1 The four groups used in the experiment; in the order they were placed in the first sheep.

4.2.4 Preparation of BMSC in Fibrin Glue

Tiseel fibrin glue (Baxter Health care Ltd, Newbury, Berkshire, UK) was reconstituted at 37°C as described in Chapter 2. Preoperatively, for cell-loaded implants, 5 x 10^4 or 5 x 10^6 caprine BMSC were counted and put into a universal container and spun in a centrifuge at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet at the bottom of the universal container resuspended into 0.25 ml of reconstituted thrombin. This was mixed with 0.25ml of the Tiseel fibrinogen component preoperatively when the fibrin –cell mixture was sprayed onto the implants. The fibrin control group consisted of fibrin glue only, without cells.

4.2.5 Surgical Procedure

All animals were handled and cared for as described in Chapter 3 (materials and methods), and the site of surgery prepared as previously described. Four skeletally mature sannan goats were used in this experiment.

Approximately six weeks prior to surgery, bone marrow aspirates were taken from goats in the BMSC group and BMSC isolated as outlined in Chapter 2 of this thesis. For inserting the tibial pins, the animal was draped to expose the medial anterior aspect of the right tibia. This area was rinsed with a chlorhexidine solution and a small (10.0 mm) incision was made 30.0 mm distal to the tibial tuberosity. The tibia was exposed by blunt dissection. A small section of the periosteum was scraped at the approximate vertical centre of the bone. A 6.0 mm hole was then drilled into the near cortex. A drill guide was inserted to centralise drilling. A 4.0 mm hole was drilled through the far cortex and tapped with an M5 tap and corresponding tap guide. The site was then washed with saline. At this point, implants in the treated groups were sprayed with fibrin with or without BMSC, according to the experimental group, onto the HA-coated

section of the implant with 1.0 atm of pressurised air. The implant was then inserted into the tibia and the threaded section screwed into the far cortex. As the implant was only 3.0 mm in diameter at the near cortex, there was a 1.5 mm gap between the implant and bone, which was bridged by the sprayed solution of fibrin with or without BMSC. On the right tibia, the top of the implant at the near cortex was routinely sealed with a square piece of e-polytetrafluoroethylene membrane (e-PTFE, Gore-Tex- Gore, Flagstaff, Arizona, USA) measuring approximately 5.0 mm x 5.0 mm, and glued onto the bone using butyl-cyanoacrylate glue (Vetbond™, National Veterinary Supplies. Talke Pits, Stoke-on-Trent, UK), after which, the fascia, muscle, and skin were sutured. Four more implants were similarly inserted in each tibia, with each implant 30 mm apart.

A spray bandage (Opsite, Smith & Nephew, Gallows Hill, Warwick, UK) was applied, before the animal was brought out of anaesthesia. All animals received 100 mg of Ceftifur antibiotic as routine prophylaxis once a day for 3 days. Animals were sacrificed 6 weeks after the procedure by intravenous injection of 0.7 mg/kg pentabarbitone (20 % JM Loveridge Ltd., Southampton, UK). Both tibiae were harvested immediately and fixed in 10% neutral buffered formalin (NBF) and processed for histology.

4.2.6 Undecalcified Hard Resin Histology and Analysis

Tibiae were prepared for histology as described in Chapter 3. A band saw was used to cut out approximately 20 mm sections of bone around each screw site from the tibiae, to facilitate processing and sectioning. Thin sections were prepared as previously described (Chapter 3, section 3.2.9) and bone area within the defect and bone-implant contact quantified using image analysis techniques.

4.2.7 Statistical analysis

To test the normality of the distribution of the data, the Kolmogorov-Smimov test was performed, using SPSS 11.0 for Mac OSX. Non-parametric data was analysed using an analysis of variance (Friedman-s test) for non-parametric related samples. Data from the different groups were compared in pairs using the Mann Whitney U test. Results were considered significant when $p < 0.05$.

4.3 Results

Goats fully recovered within a few days post-operatively, and were weight bearing on all four limbs.

4.3.1 Histology

New Bone Formation

Effect of ePTFE:

Using e-PTFE membrane increased the bone formation at both low BMSC concentration (1.0×10^5 BMSC/ml, 61.32 ± 6.94 %) and high BMSC concentration (10×10^6 BMSC/ml, 57.46 ± 4.39 %). This was statistically significant when compared to the group where no PTFE was used (1.0×10^5 BMSC/ml, 30.76 ± 9.43 %) and (10×10^6 BMSC/ml, 28.27 ± 14.64 %) with a p value of 0.03 and 0.05 respectively.

In the fibrin only groups and the group where BMSC were pre-treated in osteogenic supplemented media, presence (fibrin, 61.80 ± 5.6; Og 10×10^6 BMSC, 54.84 ± 3.98) or

absence (fibrin, 48.91 ± 9.21; Og 10 x 10^6 BMSC, 45.88 ± 14.7) of e-PTFE did not significantly increase new bone formation (p= 0.27 and 0.58 respectively).

Effect of varying cell dose:

No significant difference was noted in bone formation between $1x10^5$ BMSC (30.76 ± 9.43%) and $10x10^6$ BMSC (28.27 ± 14.64%).

Effect of osteogenic differentiation of BMSC:

Osteogenic differentiated $10x10^6$ BMSC (45.88 ± 14.7%) produced more bone than undifferentiated $10x10^6$ BMSC (28.27 ± 14.64%). However, this difference was not significant when ePTFE was used (54.84 ± 3.98% in the og $10x10^6$ BMSC group vs 57.46 ± 4.39 % in the $10x10^6$ BMSC group).

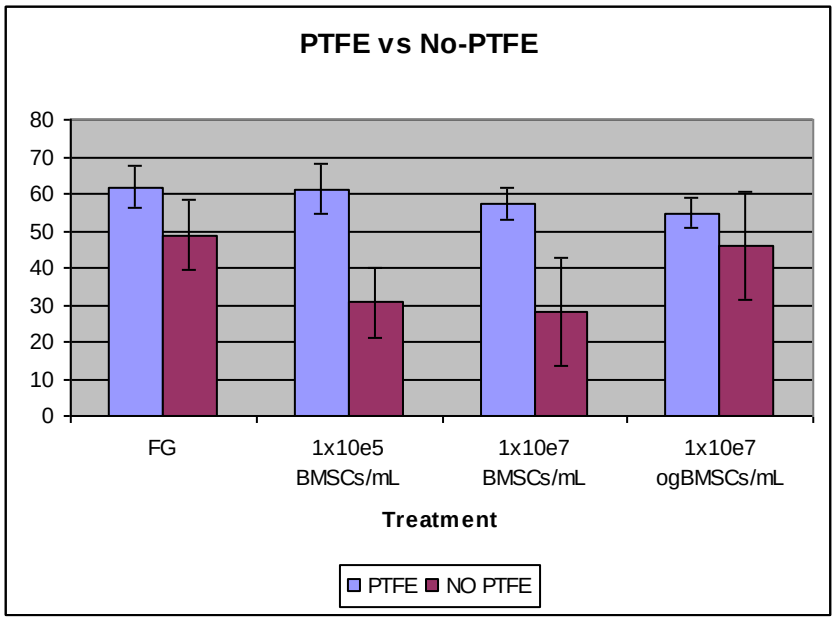
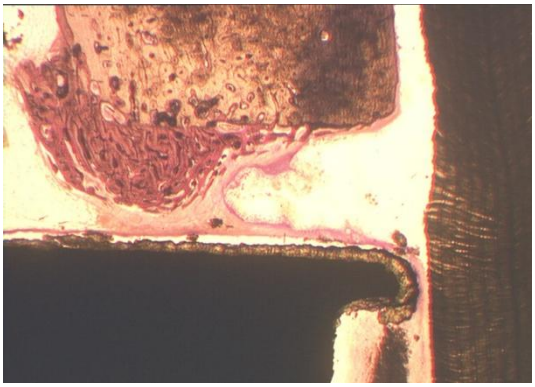
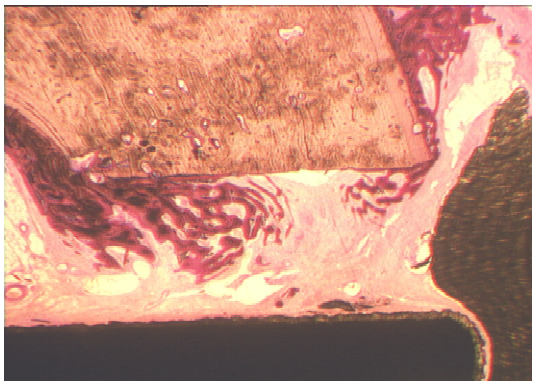


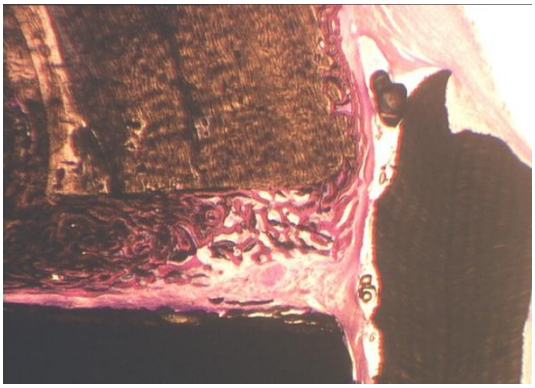
Figure 4.1Percentage new bone formation (y axis) in the four treatment groups (x axis) are compared with and without the effect of e-PTFE. Statistically significant increase in bone formation was noted with e-PTFE at 1x10e5 and 1x10e7 BMSC/mL, compared to no e

Bone Implant contact

New bone formation was noted at the endosteal and periosteal surfaces of the tibial defects. Often fibrous tissue was noted at the bone implant interface. In one sample treated with 1x10e5 BMSC/mL and in two cases treated with 1x10e7 BMSC/mL, bone implant contact was noted. The percentage bone implant contact in these samples was 39.6%, 18.4% and 44.3% respectively. No inference could be drawn from these findings.

A 

B 

C 

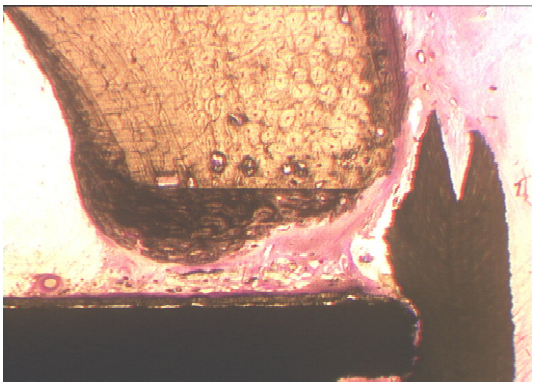
D 

Figure 4.2 Figures A, B, C&D show some typical examples of bone implant interface as seen on histological sections (4x magnification). The implant (screw) is seen at the bottom of the each image. The old cortical bone is seen at the top part of the images.

Figure 4.2 A is a sample treated with fibrin only. Figure 4.2 B is a sample treated with $1x10^5$ BMSC/mL. Figure 4.2 C is a sample treated with $1x10^7$ BMSC/mL. Figure 4.2 D is a sample treated with $1x10^7$ ogBMSC/mL. New bone formation is seen in all samples. Bone implant contact is not complete in any of these examples, with fibrous tissue noted at the implant interface.

4.4 Discussion

A careful interpretation of the results in this study is crucial, considering the various interactions that could take place between e-PTFE, BMSC and ogBMSC and fibrin.

The role of e-PTFE in guided bone regeneration is established by the increased bone regeneration noted in the e-PTFE group compared to the non e-PTFE group. This difference is less marked in the fibrin group and ogBMSC group. An untreated control was not used in this model. However, conclusions can be drawn from two previous studies where similar models were used. Kalia (2007) studied the same model in sheep and noticed the highest average new bone formation in the untreated control group. Bhumbra et al (1998) showed increased bone formation using e-PTFE without BMSC in a rabbit femur model.

There may be a few possible explanations for this including the fact that guided bone regeneration using e-PTFE is not ideal for all cell types. It may be detrimental to concentrate BMSC in a small defect using a semipermeable membrane. This might explain why the new bone formation in the no BMSC (fibrin only) group was higher than in the other groups. This may also explain the lack of dose-related increase in the new bone formation noted in the two BMSC groups; and the ogBMSC group not showing significantly higher bone formation compared to all other groups. The presence of a small defect in otherwise healthy bone should have triggered the natural bone remodelling process. Fibrin promotes cell proliferation and migration (Becker et al., 2004). Tisseel contains factor XIIIa, and fibronectin, which encourages cell migration (Cox et al., 2004); (Brown et al., 1993), in addition to increasing levels of the angiogenic factor VEGF (Hojo et al., 2003). In such a situation, the presence of BMSC may have led to cell-cell interactions with migrated cells and perhaps inhibition of one

or more group of cells, reducing the overall bone formation. This may explain why the fibrin only group by providing a natural scaffold, enhanced optimum bone regeneration. It must be noted that fibrin itself is osseoinductive only in combination with osteoprogenitor cells such as BMSC or an osteoconductive scaffold such as hydroxyapatite (Abiraman et al., 2002); (Lee et al., 2005); (Yamada et al., 2003). The increased bone growth noted in the fibrin only group in this study is probably not a direct result of its osseoinductive effect, but reflects the lesser bone formation noted in the other groups.

The model itself may be responsible for the pattern of results noted. While the screw was fixed to the far cortex, there was a 1.5mm gap between the implant and bone in the near cortex to load the BMSC with or without fibrin. This would allow micromotion when the tibia is loaded. While lower levels of micromotion are known to be beneficial for stimulating bone formation, higher motion can cause fibrous tissue formation (Jasty et al., 1997).

One marked difference between the tibial defect model and the acetabular model described in Chapter 3 or rigid fixation models described by Petite, et al. (2000) and Arinzeh, et al. (2003) is the pattern of mechanical loading of the construct. Mechanical load promotes proliferation and differentiation of osteoblastic precursor cells (Nagatomi et al., 2003), (Ignatius et al., 2005). However, the pattern of strain may decide the differentiation of the precursor cells along definitive cell lineages (Hamilton et al., 2004).

Whereas low stress and strain are conducive to intramembranous bone formation, compressive stresses have been shown to induce a chondrogenic response in BMSC. Ignatius, et al. (2005) demonstrated increasing expression of the master osteoblastic gene CBFA-1 after mechanical stretching of a human osteoblast precursor cell line

(hFOB 1.19). It has been proposed that this mechanical effect is regulated by the MAP kinase (MARK) signalling pathway, using extra cellular signal-regulated kinase 1/2 (ERK 1/2) (Simmons et al., 2004).

Thus, the cells may not have differentiated into osteoblasts or may have differentiated into another cell type such as a fibroblast phenotype.

Future studies with this model must be planned carefully. It is desirable to 'mark' cells to note migration and differentiation of implanted BMSC. Using varying defect sizes may help establish their role of implanted cell numbers on bone formation. Analysis of forces acting at the defect size can explain the pattern of mechanical stress on the BMSC. It may also be useful to use dual energy X-ray absorptiometry (DEXA) to digitally monitor and quantify bone density to analyse bone formation. Further studies in other successful models may be ideal for determining the optimum BMSC concentration to promote maximum cell growth. Characterising the BMSC using cell markers may confirm their osteogenic differentiation potential.

4.5 Conclusion

In conclusion, e-PTFE seems to play a role in guided bone regeneration using BMSC. The fibrin only group had an overall higher bone formation, probably resulting from lesser bone formation in other groups. Both interactions between fibrin, BMSC, e-PTFE and mechanical factors related to this model are responsible for these results. Simpler and more carefully planned studies are recommended in order to analyse these factors in future when the bone defect model is used.

Chapter 5

5. The Role of Differentiated Bone Marrow Stromal Cells in Augmenting the fixation of Massive EndoProsthetic Replacements

5.1 Introduction

As discussed in Chapter 2 of this thesis, BMSC can be isolated from bone marrow aspirate. When cultured in osteogenic supplemented culture media, they differentiate to form osteogenic precursor cells that show high ALP activity and can produce deposits of calcium phosphate. In the previous chapter, the role of these differentiated cells in stimulating new bone formation was studied. However, as discussed in that chapter, due to the characteristics of the small defect model and the use of PTFE membrane, interpretation of results is difficult. Besides it is not known if varying the numbers of BMSC introduced into the defect site affects the ability of the differentiated stromal cells to promote bone formation.

A massive endoprosthetic replacement in a sheep was the model chosen for this study. A similar model has previously been used to successfully demonstrate the ability of the BMSC to augment the fixation of this prosthesis (Kalia et al., 2006). Endoprosthetic replacements are increasingly used in modern surgical management of tumors of the extremities. In the lower limb, bone tumor replacement prosthesis has a significantly lower survival rate when compared to total hip replacements (Blunn et al., 2000). Aseptic loosening is a common cause of failure (Unwin et al., 1996); (Roberts et al., 1991). In the absence of sufficient bone stock revisions of the original implant, due to aseptic loosening are not as successful as a primary replacement and sometimes amputation is required. Most common endoprosthesis for long bones are fixed to the remaining bone using an intramedullary cemented stem. Increasing the bone growth and osseointegration at the implant bone interface may improve the longevity of endoprosthetic replacements. One way that this has been achieved recently is the use of a grooved hydroxyapatite coated collar that is an integral part of the shaft and

positioned at the shoulder of the implant adjacent to the transection site of the bone. A number of studies have shown that this encourages bone bridging and enhances the fixation of the implant (Whiteside and Easley, 1989); (Keaveny and Bartel, 1993). Unfortunately, bone bridging is not that predictable and occurs only in around 70% of distal femoral cases and the bone that is formed is of variable quality. Application of stem cells to the HA collar may reduce the variability of bone formation.

The hypotheses of this chapter are:

1. BMSC differentiated in an osteogenic supplemented media (ogBMSC) will improve bone growth compared to undifferentiated BMSC

2. A higher dose of ogBMSC will improve bone growth compared to a lower dose of ogBMSC.

The aims and objectives of this chapter are to:

1. Spray differentiated ogBMSC at two different concentrations (2×10^6 ogBMSC and 10×10^6 ogBMSC) in fibrin glue onto the HA-coated collars of tibial midshaft replacements in an ovine in vivo model;

2. Compare the area of new bone formation between undifferentiated (10×10^6 BMSC) and differentiated BMSC (2×10^6 ogBMSC and 10×10^6 ogBMSC).

3. Measure the area of new bone growth around the implants using radiography, and compare these results to implants treated with fibrin glue only.

4. Observe and measure the area of bone growth around each implant collar using histological techniques.

5. Measure the bone-implant contact of each implant collar.

5.2 Materials and Methods

Experimental model: Skeletally mature (65-85kg) English mules were selected as the animal model for this study. All animals were cared for as described in Chapter 2 (Materials & Methods).

5.2.1 BMSC Isolation and Expansion

BMSC were isolated as previously described (See Chapter 2, section 2.2.5 Materials and Methods). In all cases cells were expanded to passages 3 and 4 for use in this study.

5.2.2 Experimental Groups

There were four experimental groups, with six animals randomly assigned to each:

Group 1: Implants sprayed with 2 ml fibrin glue/collar.

Group 2: Implants sprayed with 10×10^6 autologous BMSC/collar in 2 ml fibrin glue.

Group 3: Implants sprayed with 2×10^6 osteogenic autologous BMSC/collar in 2 ml fibrin glue.

Group 4: Implants sprayed with 10×10^6 osteogenic autologous BMSC/collar in 2 ml fibrin glue.

5.2.3 Preparation of BMSC in Fibrin Glue

The fibrin glue used was Tisseel® fibrin glue (Baxter Health Care Ltd., Newbury, Berkshire, UK). This glue consisted of four components, a vapour-heated Tisseel® powder component (100-130 mg protein, of which approximately 75-115 mg is fibrinogen); a bovine aprotinin solution: a vapour-heated thrombin powder (45-55 mg protein containing about 500 IU^2 active thrombin), and a calcium chloride solution (see Chapter 2 for a detailed description of fibrin's properties). The Tisseel® fibrinogen component was reconstituted in the aprotinin solution after a 10-minute incubation at 37°C in a heater manufactured by Baxter Health Care (Fibrinotherm®, Baxter Health Care Ltd., Newbury, Berkshire, UK). Pre-operatively, for cell-loaded implants, the BMSC were centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet at the bottom of the universal container was re-suspended in the 1 ml of reconstituted thrombin and injected back into the manufacturer's vial for transport to the operating theatre.

5.2.4 Implant Design and Manufacture

The implant used was a mid-shaft tibial replacement, which was a design that had previously been used in a goat *in vivo* study by (Kalia, 2007) and Coathup, et al. (2000). Implants were made from titanium alloy (T16A14V). The implant shaft was 50 mm long and 10 mm in diameter. HA-coated collars were used at each transection site. HA coatings were highly crystalline and were around 50 μm thick (Plasma Biotal, Tideswell, North Derbyshire, UK). The collars were 15 mm in length, with 1 mm grooves. The implant consisted of proximal and distal parts that were joined together during surgery by two juxtapositioned screws. Each stem was fixed within the intramedullary canal using cement. A long groove was present along each stem to

promote the interlocking of cement (Figure 5.1).

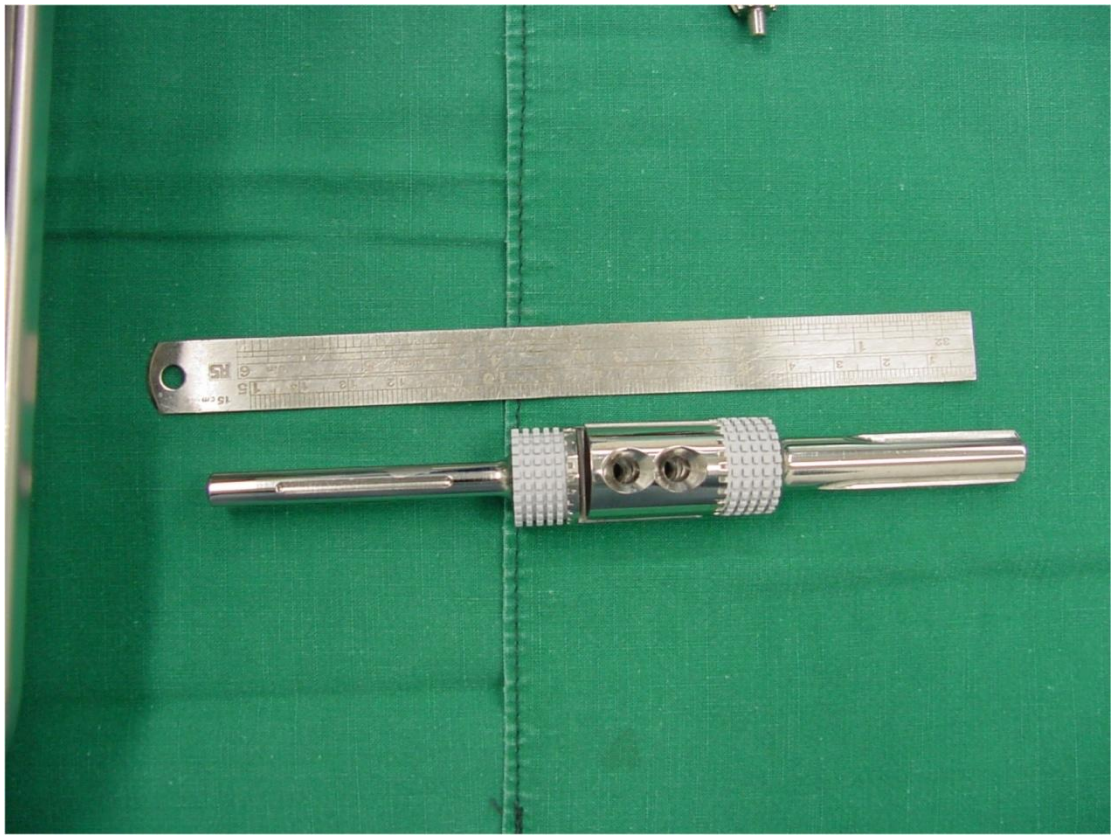


Figure 5.1 Titanium mid shaft tibial endoprosthesis with HA coated collars

5.2.5 Surgical Procedure

All animals were handled and cared for as described in Chapter 2 (Materials and Methods) and the site of surgery was prepared as previously described. Approximately six weeks prior to surgery, bone marrow aspirates were taken from skeletally mature mule ewes, from the appropriate experimental animals and BMSC isolated as outlined in Chapter 2 of this thesis.

For implantation of the tibial massive endoprosthesis, tibiae were first clipped to expose the skin, after which the area was washed with an iodine solution (as described in Chapter 2). The operation was carried out under sterile conditions. The animal's right leg was draped to expose the medial anterior aspect of the right tibia. This area was

rinsed with a chlorhexidine solution and then a proximal-distal incision 60 mm in length was made, starting 50 mm distal to the tibial tuberosity. A 50 mm section of the tibial midshaft was excised 60 mm from the tibial tuberosity using an air-saw. The periosteum from this section was removed intact.

Intramedullary canals were prepared for implantation by first removing the bone marrow, then by repeated washing with saline (a sterile, 0.9% sodium chloride solution). Where necessary, canal diameters were increased to ensure a minimum cement mantle thickness of approximately 1.5 - 2.0 mm. In experimental group animals, 2 mL of autologous BMSC in fibrin glue was sprayed onto the HA-coated surface of the implant collars (proximal and distal) at 1.0 atm of pressure. To do this, the fibrinogen and thrombin components of the fibrin glue were transported to the operating theatre with or without BMSC and maintained at 37° C in a Baxter Fibrinotherm® device (Baxter Health Care Ltd., Newbury, Berkshire, UK) until used. Each component was loaded into a 1.0 mL syringe (included in the Tisseel® package) and snapped into a dual-syringe holder with attached plunger device. To spray the fibrin glue using this system, a Duplojet® (Baxter Health Care Ltd., Newbury, Berkshire, UK) spray set was used, which consisted of sterile plastic tubing which connected to a dual-syringe tip, which allowed air to come into contact with the fibrin components as they came out of the syringe. The other end of the tubing was connected to a Fibrijet® (Micromedics, Inc., St. Paul, Minnesota, USA) pressure gauge, which was connected to a pressure pump. The Fibrijet was set to 1 atm and the plungers of the fibrin system pressed to allow release of aerolised fibrin onto the surface of the HA-coated implant collars. The implants were slowly turned as the fibrin was applied to allow an even distribution of fibrin around the collar. In the BMSC groups, the corresponding concentration of cells was applied to each collar (proximal and distal) of every implant. Control animals received fibrin glue only or no treatment at all to the collar. Implants were cemented in

place using Palacos R cement with Gentamicin (Biomet Europe, South Wales, UK). The two halves of the prosthesis were joined centrally, and the surrounding fascia, muscle and skin were closed.

A spray bandage (OpSite®, Smith & Nephew, Gallows Hill, Warwick, UK) was applied prior to recovery. Animals were allowed immediate postoperative mobilisation and weight-bearing as tolerated. Antibiotic and analgesic prophylaxis was administered daily with subcutaneous injections of Exenel™ (ceftiofur hydrochloride, 1 mL/50 kg, Pfizer Animal Health, Tadworth, Surrey, UK) and IM injections of Vetergesic™ (buprenorpine, 0.6 mg/animal, Reckitt and Colman Products Ltd., Hull, UK) for three days post-operatively. Animals were kept in individual pens until fully weight-bearing and were then grouped together in large pens.

Animals were euthanised 6 months post-operatively by an intravenous overdose of 0.7 mg/kg pentabarbitone (20, J.M. Loveridge Ltd., Southampton, UK).

5.2.6 Radiography and Radiographic Analysis

Medio-lateral (ML) radiographs of the right tibiae were taken at six months post-operatively for the fibrin control and the BMSC treated groups, using an MX4 X-ray machine (PLH Medical Ltd., Watford, UK). For analysis of new bone formation, digital images of the radiographs were captured using a JVC KY F55B Colour Video Camera using Zeiss KS300 software (Imaging Associates, Thame, UK). The radiographed area adjacent to the implants was divided into eight regions of equal size, and labelled from A to H. Images were captured using a JVC KY F55B Colour Video Camera along with Zeiss KS300 software (Imaging Associates, Thame, UK). Bone area in these regions was then quantified using the KS300 image analysis software.

5.2.7 Histology and Analysis

Following sacrifice, all right tibiae were prepared for histology by stripping excess soft tissue and fixing in 10% neutral buffered formalin (NBF) for a minimum period of one week. Specimens were dehydrated in increasing concentrations of industrial methylated spirit (IMS). Samples were de-fatted in chloroform and immersed in a 50% IMS, 50% resin mixture (LR White Resin, London Resin Company Ltd., Reading, Berkshire, UK), followed by immersion in 100% resin. Samples were then cast using a polymerisation accelerator (LR J White Accelerator, London Resin Company Ltd., Reading, Berkshire, UK). Specimen blocks were sectioned at four sites using a water-cooled band saw with a diamond-edged blade (Exact saw, Germany). One section was made through the centre of the proximal collar and one through the centre of the distal collar. Thin sections (approximately 80 µm thick) were prepared (Exact grinding machine, Germany; and Exact Polishing machine, Germany) and stained with Toluidine Blue (Bancroft and Stevens, 1986) and Paragon (Tanzer, et al., 2003) to stain soft tissue and bone, respectively. Stained slides were then viewed under an Olympus light microscope and images digitally captured using a JVC KY F55B Colour Video camera. Bone area and bone-implant contact was quantified using image analysis software (Zeiss KS300 software, Imaging Associates, Thame, UK).

Bone Area

To measure bone area using image analysis software, the area of bone was ‘encircled’ using freeform polygons and the area within it was calculated in mm^2 by the image analysis software.

Bone-Implant Contact

To measure the presence of bone-implant contact, 48 equi-distant points spanning the entire surface of the implant collar were analysed at 2.5 x magnification. The results were recorded as yes or no (0 or 1) at all points and the percentage of contact noted.

5.2.8 Statistics

To test normality of the distribution of the data, the Kolomgorov-Smirnov test was used, using SPSS 11.0 for windows. Parametric data was analysed using an independent-samples T-test; non-parametric data was analysed using the Mann-Whitney U test. Results were considered significant when $p < 0.05$.

5.3 Results

Sheep fully recovered within a few days post-operatively and were weight-bearing on all four limbs for the duration of the experiment.

5.3.1 Radiology

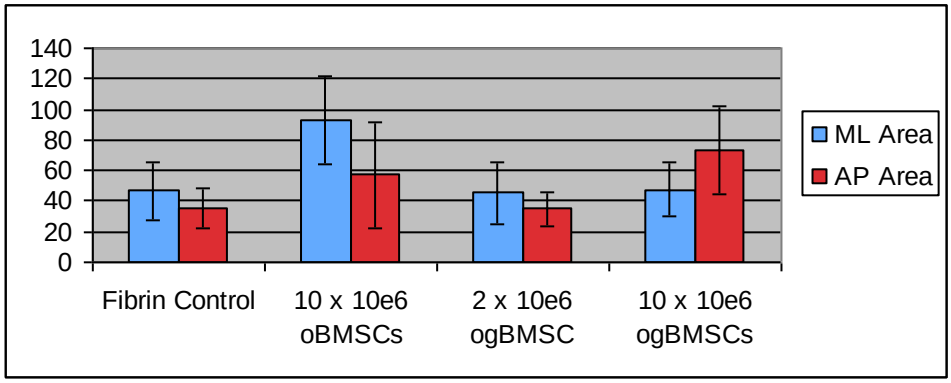


Figure 5.2 Radiographic results of medio-lateral versus antero-posterior bone growth in the study groups

There were distinct patterns of growth observed around the implants. The largest amount of bone was observed posteriorly and medially along the implant shaft. A greater amount of bone growth adjacent to the implants was observed at the proximal transection site than distally. The greater amount of new bone formation could be seen in the two BMSC-treated groups ($10xl0^6$ BMSC and $10x10^6$ogBMSC/collar) compared to the control fibrin glue-treated groups and the low dose ogBMSC ($2x10^6$ ogBMSC) group.

Bone Area

	ML Area	SE	AP Area	SE
Fibrin Control	46.84	18.81	35.16	13.39
10 x 10e6 oBMSC + fibrin glue/collar	92.57	28.94	56.94	34.69
2 x 10e6 OBs + fibrin glue/collar	45.75	20.27	34.87	10.81
10 x 10e6 OBs + fibrin glue/collar	47.68	18.08	73.42	29.04

Table 5.1 Table depicting the average area of bone formation seen on AP and ML plain radiographs at 6 months.

The area of new bone formation in the four study groups is summarized in Table 5.1. The highest increase in new bone formation was noted in the $10x10^6$ BMSC group on the ML view and the $10x10^6$ ogBMSC group on the AP view. Compared to the fibrin glue group the results were not statistically significant in any of the BMSC groups.

5.3.2 Histological Analysis

Bone Area

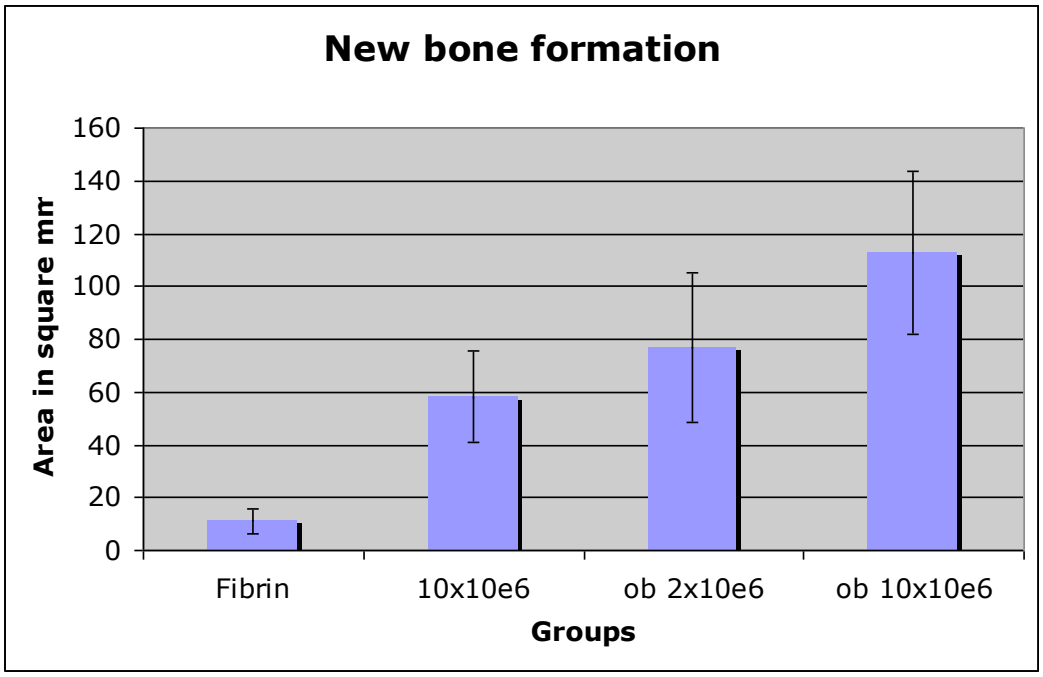


Figure 5.3 Graph showing the area of new bone formation in the four experimental groups.

In all four groups, both woven and lamellar bone was observed in and around the collars of the implants. The fibrin only control group was noted to have a layer of fibrous tissue directly in contact with the implant in most cases. In the BMSC treated groups, greater bone and lesser fibrous tissue were routinely noted. The mean area of new bone formation was noted to be highest in the high dose ($10x10^6$) ogBMSC group (112.67 ± 30.75 mm^2), followed by the low dose ($2x10^6$) ogBMSC group (mean 76.84 ± 2.25 mm^2) and finally the high dose ($10x10^6$) BMSC group (mean 58.22± 17.22 mm^2).

Compared to the fibrin treated group (mean 11.24 ± 4.75 mm^2), a significant increase in bone area was noted in histological sections taken at the mid point of the implant collars in the high dose BMSC group (mean 58.22± 17.22 mm^2 ;p = 0.02) and the high dose ogBMSC group (mean 112.67 ± 30.75 mm^2 ; p = 0.01). A greater area of bone formation was noted in the low dose ogBMSC group (mean 76.84 ± 2.25 mm^2). However, compared to the fibrin only control group, the difference in bone area was not statistically significant (p= 0.3). Comparison of the groups treated with BMSC showed a statistically significant increase in the ogBMSC group compared to the high dose BMSC group (p= 0.02). However, no statistical significance was noted in the area of new bone formation between the high dose BMSC and low dose ogBMSC (p=0.7) or the high and low dose ogBMSC (p=0.26).

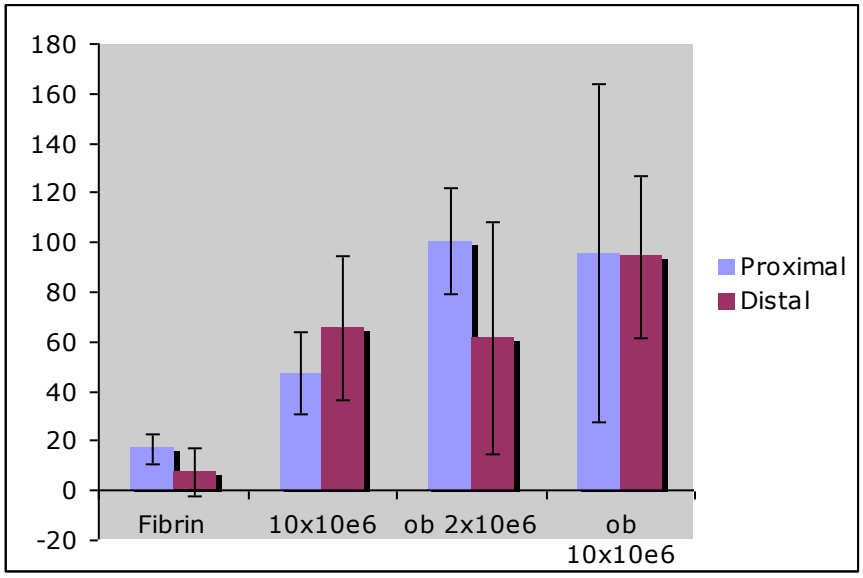


Figure 5.4 Graph comparing proximal and distal bone area in the four study groups

Comparison of the area of new bone formation around the implant collar showed no significant difference between the proximal and distal collars of the implants in any of the study groups. The mean bone area, standard error of the mean and the p values are summarized in Table 5.2.

	Mean area in mm^2	SE of mean	P value
Fibrin – proximal collar	16.89	6.11	
Fibrin – distal collar	11.2	9.66	0.67
$10x10^6$ BMSC – proximal collar	47.26	16.51	
$10x10^6$ BMSC – distal collar	65.52	29.32	0.63
$2x10^6$ ogBMSC – proximal collar	100.30	21.32	
$2x10^6$ ogBMSC – distal collar	61.21	46.93	0.50
$10x10^6$ ogBMSC – proximal collar	95.65	67.88	
$10x10^6$ ogBMSC – distal collar	95.41	32.61	0.98

Table 5.2 Comparison of proximal and distal collars in the four study groups

Bone - Implant Contact

The average bone-implant contact was noted to be 17.18% (± 7.93) in the $10x10^6$ BMSC group, 27.78% (± 10.23) in the $2x10^6$ og BMSC group and 28.13% (± 18.33) in the $10x10^6$ og BMSC group. The bone-implant contact was not found to be statistically different (p value of 0.4, 0.8 and 0.9 respectively) in any of the above BMSC treated groups compared to the fibrin only control group (21.58% ± 9.67).

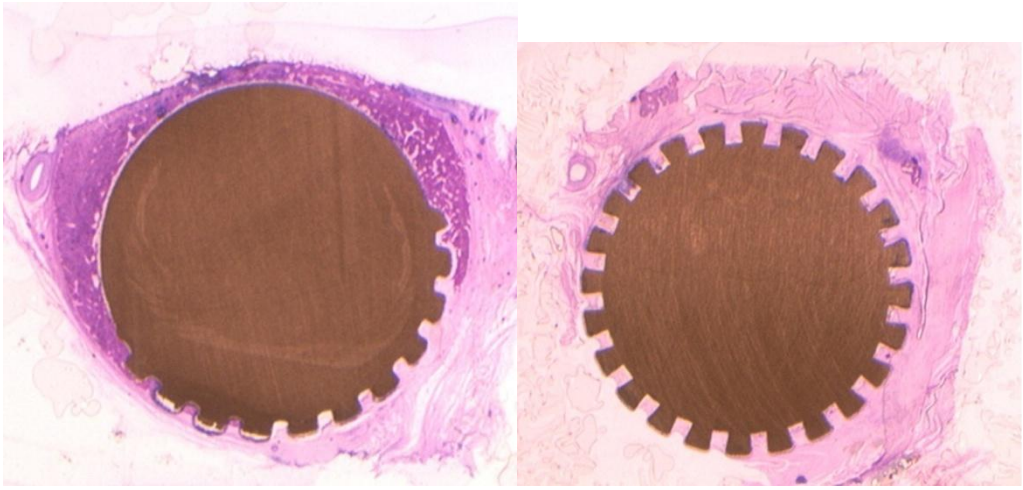


Figure 5.5 Histological sections of bone implant junction. HA-coated implant collars showed increased bone formation (left) compared to fibrin sprayed implant collars (right)

5.4 Discussion

My study has demonstrated that BMSC, when sprayed onto the surface of HA-coated implant collars, significantly increased bone formation around the collars and improved bone-implant contact to massive segmental bone tumour implants. Differentiating BMSC in osteogenic supplemented media further increased the total area of new bone formation. Promoting the growth of bone at the collar of the massive implants will aid in load transfer between the bone and implant. This may improve the longevity of fixation of the implant.

Radiographic analysis of bone growth in all groups indicated a similar pattern, whereby there was more new bone formation medially and posteriorly around the implants and less new bone formation laterally and anteriorly. This pattern is similar to that observed in humans with femoral endoprosthetic replacements, where medial and posterior increases in bone mineral density (BMD) was found to be accompanied by a decrease

laterally and anteriorly (Inglis and Walker, 1991). This reflects the pattern of stress and strain around proximal femoral replacements measured telemetrically (Taylor et al., 1997) and a study using photoelastic coatings to measure shear strain (Hua and Walker, 1992), where the strain measured medially was 53% of normal on the medial side, but only 35% on the lateral side of the implant.

In my study radiological analysis showed an increase in bone formation in all groups at 6 months. The highest increase in new bone formation was noted in the $10x10^6$ BMSC group in the ML view and the $10x10^6$ ogBMSC group in the AP view. However, no statistical difference was noted between the fibrin treated and BMSC treated groups. It is possible that plain radiographs were not sensitive to detect new bone formation. In future studies it may be possible to accurately detect new bone formation by using sensitive radiological tools such as three-dimensional reconstruction of multiple thin section computer tomography.

Histological analysis of the mid section of the massive implants showed an increased area of new bone formation in all the BMSC treated groups compared to the fibrin only group. This confirms the role of BMSC in promoting new bone formation. A statistically significant increase in new bone formation was noted in the high dose BMSC group and the high dose osteogenic supplemented BMSC group compared to the fibrin only group. In the low dose osteogenic supplemented BMSC group, there was no statistically significant increase in bone growth compared to the fibrin only group. It is possible that the beneficial effect of BMSC and the osteogenic supplemented BMSC groups is only seen above a certain cell concentration. Whether the higher cell concentration used in my study was optimal for bone formation, could not be determined from my investigation. My study also shows that, the increase in bone growth can be further improved by differentiating the BMSC in an osteogenic supplemented media.

Massive endoprosthesis are currently in clinical use. This model has obvious advantages in terms of immediate weight bearing mobilization. Fibrin glue is a convenient vector for BMSC as it is commercially available and simple to reconstitute and assemble. BMSC have also been studied in rats when combined with a gel carrier (Lee et al., 2005), or when seeded in an osteoconductive surface such as ß-tricalcium phosphate or HA, in conjunction with an internal plate (Arinzeh et al., 2003), external distraction (Richards et al., 1999) or external fixation (Lee et al., 2005). It is possible that osteoinduction could be further encouraged by using a scaffold that promotes the proliferation of cells and their differentiation into osteoblasts, as well as retaining them in the structure. An example of this could be fibrin combined with BMPs, HA or ß-tri-calcium phosphate, or all of these combined (Hong et al., 2006), (Han et al., 2005); (Schmoekel et al., 2005).

It has been suggested that fibrin glue can have a pro-angiogenic effect, which may promote new bone growth or repair (Kania et al., 1998). It is possible that in this study fibrin played some role in increasing bone formation in the fibrin-only control group. However, all the BMSC treated groups in this study have shown a higher average new bone formation above the fibrin treated group. This provides evidence for the bone promoting role of BMSC.

HA has already been well documented in the literature as being osteoconductive (Blunn et al., 2000); (Coathup et al., 2000); (D'Antonio et al., 1992), (D'Antonio et al., 1992, Geesink, 1993). Possible mechanisms of this bone-promoting effect have been suggested. One theory is that as HA slowly degrades in the body, the degradation products released from the implant surface, such as calcium and phosphate, or the change in pH (Ozawa and Kasugai, 1996); (Kurioka et al., 1999) may promote differentiation of the BMSC to osteoblasts, or even improve bone formation by host osteoblasts which have migrated to the implant site, or the BMSC-derived osteoblasts

(Datta et al., 2006). Kilpadi, Chang and Bellis (2001) have also shown that HA has the capacity to bind proteins such as important integrins (for example a5ßl and avß3), as well as fibronectin, vitronectin and osteoblast precursor cells, when compared to metals.

Integration of an implant into the bone shaft is important for better load transfer to the bone shaft and off-loading the implant stem, which may reduce loosening of the implant. In patients with thin stems, this may reduce mechanical failure. This may be important in younger patients, where a relatively small implant is inserted. The osseointegration of the implant may allow these implants to survive into adulthood.

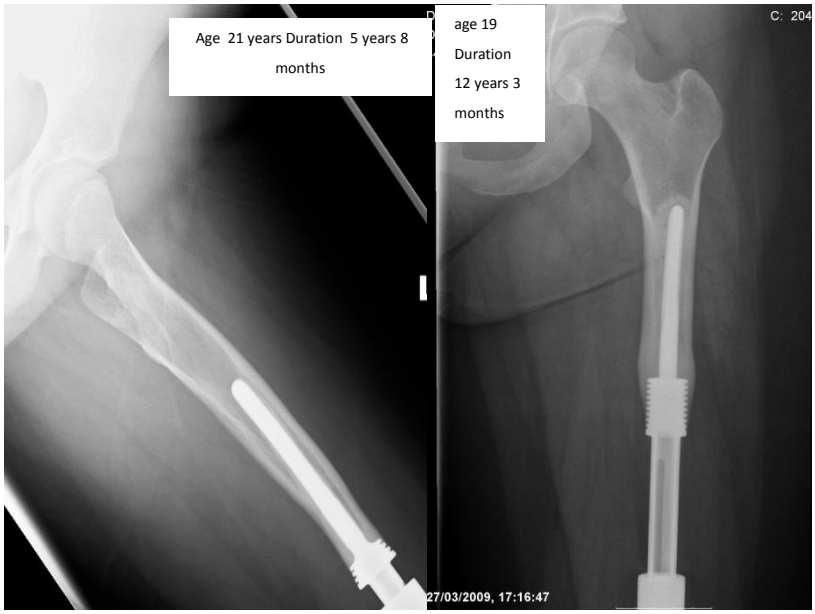


Figure 5.6 Radiograph of a patient with a relatively thin stem inserted when the bone was still growing. The incorporation of the HA has probably protected the implant from loosening and fracture despite the relative bone growth.

One drawback of using autologous BMSC in a patient with a malignant bone tumor is the theoretical question of whether the isolated BMSC have metastatic cells. The clinical solution to this would be to use allogenic BMSC (Horowitz et al., 1993).

5.5 Conclusion

In conclusion, in an in vivo massive endoprosthesis model, $10x10^6$ BMSC increases the area of new bone formation. This can be further increased by treating the BMSC in an osteogenic supplemented media.

Chapter 6

6. Discussion

My thesis confirms that BMSC suspended in fibrin glue can be sprayed onto the surface of orthopaedic implants to improve osseointegration by promoting new bone formation adjacent to the implants and decreasing fibrous tissue in contact with the implants. The hypothesis has been tested in three different implant models – a goat acetabular cup model; a sheep tibial pin model and a sheep massive tibial endoprosthesis model.

Chapter 2 describes the technique of isolating BMSC from iliac crest aspirate. Cells were expanded in culture. The cells were characterised by differentiating them down different cell lineages (osteoblastic, chondrogenic and adipogenic), using chemical cues. In order to fully characterise the oBMSC used in this study and identify them as being the multipotent cells described in the literature (Haynesworth et al., 1992); (Jaiswal et al., 1997), more in vitro work needs to be done looking at gene expression after stimulation with supplements for differentiation. Also, surface marker analysis needs to be carried out, with a panel of antigens that are known to positively or negatively react with BMSC, using a FACS or MACS system (Pittenger et al., 1999).

BMSC are thought to undergo osteogenic differentiation via a MAP kinase-mediated mechanism (Jaiswal et al., 2000). Pre-differentiation of these cells into osteoblast precursor cells or osteoblasts may help expedite the bone healing process by skipping stages of differentiation, or the requirement for certain environmental/mechanical cues in order to differentiate and, once integrated into the repair site, could begin to produce bone matrix and matrix mineralisation without delay. Additionally, the applied cells could be genetically modified to express osteogenic growth factors and cytokines.

Also described in Chapter 2 is the technique of coating the implant surface with BMSC prior to implantation. The viability, proliferation, and morphology of BMSC did not alter in vitro after being sprayed at 1 atm with fibrin glue. The advantage of using this

technique is the ability to intra operatively spray the implants with the BMSC-fibrin glue mixture. The use of such a system would be a much more convenient and translatable method, as the cells are cultured separately and then combined with fibrin per-operatively rather than pre-culture of the cells onto the implant. Pre-sterilised implants of any desired type can be used and there is no handling of implants before the intended procedure. Studies comparing ceramic cubes seeded with stem cells and then cultured with similar cubes where the cells were incorporated at the time of surgery showed that there was no difference in bone formation when the cubes were implanted in an ectopic site (Kruyt et al., 2004).

The ability of BMSC to improve bone implant contact was investigated in Chapter 3. A goat (in vivo) total hip arthroplasty model was used in this study. Culture expanded BMSC ($10x10^6$ cells) suspended in fibrin glue was sprayed on the surface of hydroxyapatite-coated press-fit acetabular cups and this was compared to fibrin only sprayed control groups. The area of new bone formation adjacent to the implants was increased when implants were treated with BMSC in fibrin glue when compared to fibrin only treated implants. Overall a 30% increase in new bone formation was noted when acetabular cups were treated with BMSC. Along the surface of the cup, the contact between bone and the implant was noted to be increased when cups were sprayed with BMSC. This increase was statistically significant at the periphery of the cups ($p=0.014$). Additionally, the BMSC treated surface also decreased the fibrous tissue in immediate contact with the implant, with a statistical difference noted at the periphery of the cup ($p = 0.09$). It is important to note that no difference in loading was observed between the study and control groups in any of the animals. The exact reason for statistically significant improvement in bone-implant contact at the periphery of the cup compared to the central portion of the cup is not clear. As outlined in the discussion section of Chapter 3, it is possibly a combination of factors including differential

loading at various points on the cup surface, cell migration and differentiation. Further studies using markers to track the cells and close measurement of forces across various points of the implant-bone construct may provide useful information regarding this phenomenon. Radiological imaging using radioisosteriometry (RSA) may be another useful measure of implant migration and its consequence on osseointegration.

In Chapter 3, a primary hip arthroplasty scenario was investigated. It would be beneficial to apply this principle in a revision hip arthroplasty scenario. At revision hip arthroplasty there is less available bone stock due to lysis. In this situation, implants coated with stem cells and fibrin glue may allow the stem cells to concentrate in the bone defects at the implant interface. In addition, if bone grafting is necessary for structural support during revision arthroplasty surgery, stem cells may be used along with the morselised bone graft.

Following on from the results of Chapter 4, another large animal in vivo model was used in order to establish if varying cell doses; differentiating BMSC using osteogenic supplements or concentrating the cells using an artificial semi-permeable membrane (e-PTFE) effects bone formation. Four groups (with or without e-PTFE) were tested in Chapter 4, fibrin only, $1.0x10^5$ BMSC, $10x10^6$ BMSC and $10x10^6$ osteogenic supplemented BMSC, forming a total of eight experimental groups. HA-coated transcortical screws were coated with one of the eight treatments, and implanted into 1.5mm tibial gaps in the mid-shaft of sheep. After a period of six months, bone formation, and bone-implant contact was assessed histologically. Using e-PTFE statistically increased bone formation in low and high BMSC group ($p= 0.03$ and 0.05 respectively) but not in the osteogenic supplemented BMSC group. Overall, no significant increase in bone formation was noted in any of the groups compared to the fibrin-only control group. One of the salient features responsible for the pattern of results noted in this study is the lack of cell loading in the tibial defect – pin model.

This, along with the absence of an untreated control group and complex interactions between the fibrin, BMSC and e-PTFE make interpretation of the results of this experiment difficult.

Chapter 5 investigated the ability of BMSC to augment new bone formation adjacent to massive orthopaedic implants in an ovine model. The effect of osteogenic supplementation of different cells doses of BMSC was also investigated. Once again, fibrin sprayed control groups were used as controls to test $10x10^6$ BMSC, $2x10^6$ osteogenic supplemented BMSC and $10x10^6$ osteogenic supplemented BMSC sprayed on the collars of massive tibial endoprosthesis. The area of bone adjacent to the implants and to the implant collars was increased when implants were treated with low and high doses of oBMSC in fibrin glue when compared to fibrin treated implants. Increasingly greater bone formation was noted with $10x10^6$ BMSC, $2x10^6$ osteogenic supplemented BMSC and $10x10^6$ osteogenic supplemented BMSC respectively. However, the results were found to be statistically significant in the $10x10^6$ BMSC ($p=0.02$) and the $10x10^6$ osteogenic supplemented BMSC ($p=0.01$). Those implants treated with a higher density of differentiated cells showed an increase in new bone formation when compared to those treated with the lower number of cells. Compared to fibrin treated groups, no increase in bone implant contact was seen in any of the BMSC treated groups. It is possible that fibrin acted as a scaffold and promoted migration and concentration of osteoblasts at the implant collar. A comparison group with untreated implants would have further highlighted this aspect.

Summary of the results of all chapters

1) Autologous BMSC can be isolated from BM aspirate and expanded along the osteogenic pathway. They can be sprayed on to the surface of orthopaedic implants intra-operatively without affecting cell viability, metabolism or proliferation.

2) Bone formation was higher with osteogenic $10x10^6$ BMSC (112.67 ± 30.75 mm^2) compared to osteogenic $2x10^6$ BMSC (76.84 ± 2.25 mm^2). No significant difference was noted in bone formation between undifferentiated $1x10^5$ BMSC (30.76 ± 9.43%) and undifferentiated $10x10^6$ BMSC (28.27 ± 14.64%).
3) Osteogenic differentiated $10x10^6$ BMSC (112.67 ± 30.75 mm^2) produced more bone than undifferentiated $10x10^6$ BMSC (58.22 ± 17.22 mm^2).
4) Using semipermeable barriers resulted in significantly increased bone formation when undifferentiated $1x10^5$ BMSC (61.32 ± 6.94% vs 30.76 ± 9.43%) or undifferentiated $10x10^6$ BMSC (57.46 ± 4.39% vs 28.27 ± 14.64%) was used. This difference was not noted when osteogenic differentiated $10x10^6$ BMSC was used.

6.1 Future Work

Further progress in this research is necessary in following directions:

1) The optimum cell concentration of BMSC has to be further established using carefully planned *in vivo* experiments.

2) The use of allogenic BMSC in place of autologous BMSC has to be investigated. One must consider that when using the autologous BMSC of a bone cancer patient, there is a theoretical risk of undetected metastatic tumour cells being isolated from the bone marrow in addition to BMSC. If this is the case, use of donor-derived, allogeneic BMSC may also be useful (Horowitz et al., 1993). However, one must also consider that all BMSC, autologous or allogenic, are immunosuppressive and express VEGF, which is essential to tumour growth (Aggarwal and Pittenger, 2005); (Ferrara and Davis-Smyth, 1997). These effects may have negative consequences for tumorigenesis in any patient (Djouad et al., 2003).

3) Most importantly, human clinical trials have to be conducted to establish the role of BMSC in augmenting the fixation of orthopaedic implants in clinical scenarios such as revision surgery, complicated trauma surgery and tumor surgery. Animal models do not always replicate complex clinical scenario. For example, during revision hip arthroplasty, extensive trauma or reconstruction following extensive tumor surgery, the surgeon is faced with the challenge of inadequate bone stock, infection and deficient soft tissue cover. Cost and ethical issues prevent development of a model that would replicate this scenario. Only human trials can provide establish the role of BMSC in these complex situations.

4) In future work, cells should be tracked to try and identify their contribution to new bone formation in *in vivo* models. This could be done by genetically modifying the cells to express a gene, such as lacZ, which encodes the bacterial enzyme P-galactosidase, green fluorescent protein (GFP) and Flue, which encodes firefly luciferase (Zhou et al., 2005).

5) A further strategy to improve bone formation around the implants could be to incorporate growth factors into the fibrin-cell mixture that is sprayed onto the HA collars. Inclusion of growth factors such as BMP-2 or BMP-7, well documented osteogenic factors, could further increase bone formation by influencing the differentiation of the cells. Alternatively, these factors could be supplied using a cell therapy approach, where genetically modified BMSC, expressing BMPs or other relevant genes, could be implanted at the repair site (Tsuchida et al., 2003); (Lieberman et al., 1999); (Edwards et al., 2005); (Mistry and Mikos, 2005). Growth factors targeting neo-angiogenesis and revascularisation could also play an important role, such as VEGF (Geiger et al., 2005); (Peng et al., 2005) and SDF-1.

6) In a clinical trial, robust clinical outcome measurements are prudent to demonstrate any increase in bone formation compared to controls. Implant migration measured using

radiostereometric analysis (RSA) may be the ideal primary outcome measure. Secondary outcome measures that can be employed may be bone density measurements adjacent to the implant as quantified using DEXA scanning, radiological assessment investigating and quantifying signs of implant loosening and function of the hip; that is, pain-free mobility as assessed using a Harris Hip Score.

7) BMSC were used in the *in vivo* models in this study, rather than peripheral bone marrow stromal cells (PBSCs). BMSC have been used in other models previously, but there is only one other described study in the literature utilising PBSCs (Wan et al., 2006). Most literature on PBSCs (Rochefort et al., 2006); (Mansilla et al., 2006); (Khosla and Eghbali-Fatourechi, 2006); (Kassis et al., 2006) have been published relatively recently, although they show a similar potential for use in bone tissue engineering applications as BMSC (Wan et al., 2006).

In conclusion, viable BMSC can be successfully isolated from bone marrow aspiration, differentiated along the osteogenic pathway and sprayed on the surface of various orthopaedic implants to improve bone-implant contact. This may be an ideal alternative to improve osseointegration of implants in challenging clinical scenarios with deficient bone stock. Moreover, this research may pave the way for further advances in tissue bioengineering such as the use of allogenic cells in immunodeficient individuals undergoing orthopaedic replacement procedures or the use of cells in combination with BMPs to enhance implant fixation. It is crucial to test the results of these studies in various clinical scenarios by using carefully planned blinded randomized control trials.

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