The development of a small animal model of wear debris induced osteolysis.

by

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The candidate confirms that the work submitted is his own and appropriate credit has been given to the work of others.

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ABSTRACT

Wear debris that is generated at the articulating surfaces of a total joint replacement is thought to play a crucial role in the inflammatory process and osteolysis that has been observed in peri-prosthetic tissue around total hip replacements. Osteolysis can lead to eventual failure of the implant and necessitate revision surgery that is associated with morbidity and mortality to patients. It is the cellular response by the macrophage that is believed to play a major role by the production of pro-inflammatory cytokines and osteolytic mediators following the phagocytosis of particulate wear debris. These findings have lead to an increased interest in the use of pharmaceutical agents to inhibit the production of cytokines and enzymes during the cascade of inflammatory process that takes place with the phagocytosis of particulate wear debris.

This study investigated the possibility of developing an *in vivo* small animal model of wear debris induced osteolysis. This model could then be used to screen potential pharmaceutical agents such as matrix metalloproteinase inhibitors. An optimal model should allow rapid multiple testing and be cheap and simple. Urinary bone markers such as N-Telopeptide and Deoxypyridinolone were investigated to examine whether these metabolites can be used in this model to monitor osteolysis. These markers can then be used to monitor the treatment of osteolysis with pharmaceutical agents. The presence and quantification of matrix metalloproteinase in this model was also investigated. Matrix metalloproteinases are believed to play an important role in osteolysis. Pharmaceutical agents such as simvastatin and doxycycline are known matrix metalloproteinase inhibitors and these agents could be screened in an *in vivo* small animal model of osteolysis.

Results from this study showed that the implantation of clinically relevant ceramic particles into the medullary canal of radiolabelled murine femora caused a significant reduction in scintillation count. However, when compared with sham surgery this difference became irrelevant and no significant osteolysis was observed. The results from the urine bone markers, both N-Telopeptide and Deoxypyrinidolone showed that these markers were not elevated in animals that were implanted with ceramic particles compared to sham surgery. There was no correlation between the bone urine markers and scintillation count of the femora that underwent implantation of ceramic particles. The results from the staining of bone for matrix metalloproteinase-9 also showed a significant difference between the animals that were implanted with ceramic particles compared to sham surgery There was no increase in the quantity of matrix metalloproteinase-9 over time in the femora that underwent implantation of particles but an increase in the number of macrophages and osteoclasts in these femora compared to controls..

In conclusion, this study has demonstrated that the implantation of clinically relevant ceramic particles into murine femora was able to induce an inflammatory response but unable to detect osteolysis and thus the *in vivo* small animal model was unsuccessful. The above study shows that even a high concentration of clinically relevant ceramic wear debris was not able to influence the scintillation counts of radiolabelled bone and the production of osteolytic mediators *in vivo*. The results of this study will hopefully prompt new questions about the biological reactions to ceramic wear debris and the possibility of developing an *in vivo* model of osteolysis.

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LIST OF ABBREVIATIONS

BCE	Bone Collagen Equivalents
$^{45}CaCl_2$	Calcium Chloride
Co	Cobalt
CoCr	Cobalt Chrome
CPM	Counts Per Minute
CSE	Control Standard Endotoxin
CSF	Colony Stimulating Factor
DAB	Diaminobenzidine
DBS	Division of Biomedical Services
DPD	Deoxypyridinoline
DPX	Dibutylphthalate Xylene
EDX	Energy Dispersive X-ray
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EU	Endotoxin Units
FGF	Fibroblast Growth Factor
H & E	Haematoxylin and Eosin
HCl	Hydrochloric Acid
HIP	Hot Isostatic Pressing
HRP	Horse Radish Peroxidase
IL	Interleukin
LAL	Limulus Amoebocyte Lysate

MBq	Mega Becquerel
M-CSF	Macrophage-Colony stimulating factor
MMP	Matrix Metalloproteinase
NTx	N-telopeptide
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PE	Polyethylene
PGE_2	Prostaglandin E_2
PMMA	Polymethymethacrylate
PMN	Polymorphonuclear Leucocytes
PSM	Pseudosynovial Membrane
PTFE	Polytetraflouroethylene
RANK	Receptor activator of NFκ-B
RANKL	Receptor activator of NFκ-B ligand
RNA	Ribonucleic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TBS	Tris Buffered Saline
TEM	Transmission Electron Microscopy
TGF	Transforming Growth Factor
THR	Total Hip Replacement
Ti	Titanium
TIMP	Tissue Inhibitor Matrix Metalloproteinase

TNF	Tumour Necrosis Factor
TRAP	Tartrate Resistant Acid Phosphatase
UHMWPE	Ultra High Molecular Weight Polyethylene
ZrO_2	Zirconium Dioxide

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CHAPTER 1

INTRODUCTION

1.1 THE HISTORY OF TOTAL HIP REPLACEMENT

Throughout England, approximately 40,000 total hip replacements (THR) are performed each year. This procedure is most commonly indicated for degenerative hip joint disease caused by osteoarthritis. Other indications include rheumatoid arthritis, avascular necrosis, post-traumatic arthritis and juvenile rheumatoid arthritis (Lim *et al.*, 1999). The operation has dramatically enhanced the mobility and quality of life of many people allowing independent living to those who would otherwise suffer substantial chronic disability. New technology involving prosthetic devices for replacement of the hip, along with advances in surgical techniques, have diminished the risks associated with the operation and improved the immediate and long term outcome of hip replacement surgery.

The first total hip replacement is attributed to Gluck in 1890. The prosthesis consisted of a carved ivory ball and socket, which was fixed *in situ* with bone glue composed of rosin, pumice powder, and plaster of Paris (Gluck, 1890). Following Gluck's experimental prosthesis, Sir Robert Jones used a femoral head made of pure gold in 1895. In 1902, Jones utilised an ivory femoral head articulating with a gold leaf lining the acetabulum. The outcomes of these early attempts of hip replacement were poor due to the high stresses across the hip joint with materials unable to tolerate such stresses (Jones, 1908).

Smith-Peterson used a glass acetabular cup in the 1920s. This glass cup was prone to continual fracture when used due to its brittleness (Smith-Peterson, 1939). Other materials used include Vitallium (an alloy consisting of cobalt, chromium

and molybdenum) for both the femoral and acetabular components, which proved to be successful and was used up to the late 1950s (Thompson, 1952). Acrylic was also utilised but this was associated with a heavy wear rate then an inflammatory reaction to the wear particles causing bone loss around the prosthesis (Judet *et al*, 1954).

All these early hip prostheses and subsequent poor outcomes, prompted new research into the development of a total hip replacement (THR). In 1938, Wiles implanted a stainless steel prosthesis into six patients with Still's disease. The femoral component was fixed to the neck of the femur by a nickel plate, whereas, the acetabular component was anchored to a buttress plate by nickel plated screws. Fixation of the hip replacement proved to be problematic and many of the prostheses were affected by loosening and failure (Wiles, 1958). The use of cobalt-chrome alloy by McKee during the 1950 to mid 1960 proved to be a reasonable and competent design. The prosthesis was secured by pinning the acetabulum and an uncemented metal stem inserted into the femoral canal to secure the femoral head (McKee & Watson-Farrar, 1966).

Throughout the history of THR, one man stands out as the pioneer in the field of hip replacement; Sir John Charnley. He believed that science could provide a solution to the problem of early loosening. Sir John Charnley started his work on reducing friction and increasing lubrication in artificial hip joints. His answer was the low frictional torque arthroplasty concept, "torque loading at the cement-bone

interface is lowest when a small diameter femoral head is articulating within the maximum outer diameter of the socket" (Charnley, 1961).

The first material used by Charnley was the polymer polytetrafluoroethylene (PTFE) which was used to replace the acetabulum. This was paired with a stainless steel femoral head and the combination possessed a very low coefficient of friction. Unfortunately, femoral osteolysis and loosening occurred after implantation of this prosthesis and most had to undergo revision after only one year (Charnley, 1968). With the same bearing materials, Charnley then used dental cement as a grout for femoral fixation and a smaller femoral head for reduced frictional torque. Unfortunately, after promising early results, severe problems arose including profound wear, loosening and granulomas in the periprosthetic tissue. There was a 95% failure rate in 300 consecutive operations along with a 10% infection rate. The problems were attributed to the high wear rates of the PTFE *in vivo*. The presence of PTFE wear debris produced at the bearing surfaces in tissues local to the implant were believed to have caused the granulomas (Charnley, 1979).

In 1962, Charnley was introduced to a new polymer, ultra high molecular weight polyethylene (UHMWPE). This material possessed a higher resistance to wear compared to PTFE and had a low friction coefficient. Later in the same year, the first UHMWPE acetabular component was implanted in conjunction with a stainless steel femoral component (Figure 1.1). Results were better than the previous prosthesis, although wear was still a problem (Charnley, 1979).

Figure 1.1

The Charnley total hip replacement

Currently, there is an interest regarding the use of highly cross-linked UHMWPE as an articulating surface for THR. Simulator studies have indicated that the wear of UHMWPE is substantially reduced by increased cross-linking (Wroblewski *et al*, 1996; McKellop *et al*, 2000). These studies have shown no measurable wear after five to twenty million cycles. The degree of cross-linking in UHMWPE is achieved by controlling the dose of radiation to which the polymer is exposed under specific processing conditions. Highly cross-linked UHMWPE is obtained

with the use of a minimum of 50 kGy of gamma radiation. Cross-linking is known to change the molecular structure of polyethylene. Bradford *et al.* (2004) investigated the *in vivo* wear mechanisms in twenty four retrieved highly cross-linked polyethylene acetabular liners in order to determine if early *in vivo* wear behaviour is accurately predicted by hip simulator studies. The average age of the patients was 60 years and the average time *in vivo* was 10.1 months. The liners exhibited signs of surface damage that had not been predicted by *in vitro* hip simulator studies. The discrepancy between *in vitro* and *in vivo* wear patterns may be due to variability *in vivo* lubrication and cyclic loading or may represent early wear mechanisms that are not well represented by long-term *in vitro* simulator studies.

Despite all these advances in surgical technique and prosthetic design, the Charnley THR continues to produce outstanding results. Callaghan et al (2004) prospectively reviewed the results of a single surgeon series of primary Charnley THR performed with cement. Twenty seven patients (10.3% of 330 hips in the initial study group) were alive at a minimum of thirty years postoperatively. The original patient series consisted of 134 women and 128 men (330 hips) with an average age of 65.1 years at the time of surgery. A Charnley stainless steel stem with a polished finish 22 mm nonmodular head and an ultra-high molecular weight all-polyethylene cup were implanted. Revision because of aseptic loosening of the acetabular and femoral components was performed in eight and three hips respectively. This study demonstrated remarkable durability of the

cemented Charnley THR over a span of thirty years with 88% of the original prostheses intact at the time of final follow-up or patient death.

In another series by the same senior author, the twenty five year results after cemented Charnley THR in patients less than fifty years old is reported. The original cohort consisted of 69 patients that underwent 93 THRs, 35 were women and 34 were men. The average age at the time of the index operation was 42 years. A stainless steel femoral stem with a 22mm diameter head and an UHMWPE acetabular component were inserted. The study reviewed 42 of the 43 living patients. The combined prevalence of radiographic failure or revision because of aseptic loosening was 13% for the femoral components and 34% for the acetabular components. The study demonstrated good durability of the cemented Charnley THR in young patients with only 5 % requiring more than one revision replacement (Keener *et al*, 2003).

Technological advances in the design of hip prostheses combined with refinement of surgical techniques have improved outcomes and decreased the risks associated with total hip replacement. However, specific questions regarding which prosthetic design and materials are the most effective for patient groups and which surgical technique yields the best outcome are still to be answered.

1.2 THE COMPLICATIONS OF TOTAL HIP REPLACEMENT

A major problem associated with THR is aseptic loosening and failure of the implanted prosthesis. This is the commonest cause of long term failure in joint replacement with 8 to 20% of patients demonstrating evidence of loosening within 10 years (Jacobs *et al*, 1994). As a result, many patients will require complex revision surgery which is not only technically challenging but is associated with poorer results than primary surgery and shorter duration of survival of the joint replacement (Callaghan *et al*, 1985). Revision surgery procedures are also associated with higher morbidity and costs (Wright & Goodman, 1996). The only current treatment option for aseptic loosening is revision surgery or suffering the symptoms and complications. The complications include progressive pain, immobility and fractures through the areas of osteolysis. Other complications of THR include bleeding, infection, deep venous thrombosis and pulmonary embolus, dislocation, leg length discrepancy and neurovascular damage.

In the past, patients between 60 and 75 years of age were considered to be the best candidates for THR. However, younger and more active patients suffering with hip disease due to osteoarthritis and other causes who would also benefit from a hip replacement are demanding joint arthroplasty. This has led to research into factors that affect the failure of prostheses and has initiated interest from industry into different bearing materials that aim to eliminate the problem of wear debris induced osteolysis and aseptic loosening.

1.3 OSTEOLYSIS AND ASEPTIC LOOSENING

Charnley noticed areas of bone loss around the implanted prosthesis which he initially attributed to infection (Charnley, 1979). Willert and Semlitsch (1977) were the first to suggest that the phenomenon was caused by a macrophage response to wear debris which eventually led to loosening of the prosthesis. They studied 123 specimens of tissues from prostheses revised due to loosening. They noticed a granulomatous tissue surrounding the implants containing a high density of macrophages and giant cells. They suggested that the osteolytic reaction may have been caused by the interaction of these macrophages with particles of polyethylene, metal and polymethymethacyrlate (PMMA) cement. When the volume of wear debris overwhelmed the lymphatic system, a chronic granulomatous reaction would occur that potentially resulted in bone lysis.

In the 1980's, osteolysis and the occurrence of a granulomatous response in tissues around THRs were attributed to a biological response to fragmented particles of the cement utilised for prosthetic fixation. The term "cement disease" was used to describe osteolysis in association with cemented implants (Jasty *et al*, 1986). This then led to an interest in the use of cementless prostheses, but the use of these designs failed to reduce the incidence of osteolysis. Studies have shown that similar reactions occur around uncemented implants, and the condition may be more appropriately called "polyethylene disease". The biological response to polyethylene particles is proposed as the major cause of aseptic loosening of implanted hip prostheses (Boynton *et al.*, 1991). There is a direct relation between the volumetric wear rate of the UHMWPE acetabular cup and the incidence of

osteolysis. Oparaugo *et al.* (2001) analysed eight reports of polyethylene wear and osteolysis in THRs and found that as volumetric wear rate in the prosthesis increased, the incidence of osteolysis and revision rate increased.

Although the natural history of osteolysis and its origin are not completely understood, its development is perceived to be related to the amount and size of polyethylene particles generated from the bearing surfaces. Linear and volumetric wear rates are the two most common measurements used to quantify polyethylene wear *in vivo* radiographically (Martell and Berdia, 1997). Although the linear wear rate is generally more accepted and easily calculated, volumetric wear represents a more accurate measurement of the amount of wear particles generated.

Orishimo *et al* (2003) investigated the relationship between the prevalence of osteolysis and both linear and volumetric wear. Polyethylene wear and osteolysis were examined in fifty-six hips after seven years of follow-up (cementless cup with polyethylene liner and 28mm modular femoral head). Osteolysis was found in twenty-three hips. The hips with osteolysis had significantly higher linear and volumetric wear rates than the hips without osteolysis. The authors in this study were able for the first time to quantify the relationship between polyethylene wear as determined by both linear and volumetric wear and the prevalence of osteolysis. The results of their analysis provided quantitative support that a wear rate of 0.2 mm/yr represents a "critical threshold" for the development of osteolysis.

1.4 THE PSEUDOSYNOVIAL MEMBRANE

The tissues from revised UHMWPE-metal prostheses show a characteristic pathology. Histological studies of tissues from patients undergoing revision procedures have revealed a periprosthetic pseudo-membrane rich in macrophages and multi-nucleated giants cells associated with polyethylene wear particles surrounding the implanted prosthesis (Goldring *et al.*, 1993; Jiranek *et al.*, 1993). It is also named a pseudo-synovial membrane (PSM) because of it's resemblance to normal synovial membrane (Revell *et al.*, 1989). This pseudo-synovial membrane is believed to be a normal response by the body to effectively wall off the implant in a wound healing response (Pizzoferrato *et al.*, 1991). Bullough *et al.* (1988) suggested that this membrane was usually complete within 2 years of the primary hip replacement.

Immunocytochemical analysis of the PSM has revealed a complex cellular structure. A layer of cells termed type A and B synoviocytes, possessing no basement membrane lines the synovial membrane that surround a joint. Studies have shown that this synovial membrane is a highly ordered structure with a deep layer of fibroblast and an overlying layer of macrophages. The same configuration of fibroblasts and macrophages within the pericellular matrix is demonstrable next to the implant. Cells in close contact to the implant stain positive for macrophage markers. Close to the macrophages are cells that stain positive for fibroblast and type B synoviocyte markers. Other cells positively identified in the PSM include CD-4 T lymphocytes (Revell *et al.*, 1997).

Korovessis and Repanti (1994) examined tissues from around revised cemented UHMWPE-metal THRs using histological techniques. The authors did not find any difference histologically between tissues from loosened and non-loosened implanted prostheses. Observations characteristic of both groups were the presence of a synovial-like membrane around the implant, consisting of aggregates of macrophages and giant cells within a dense fibrous matrix. The most common particle type observed was cement, but both UHMWPE and metal particles were observed in most tissues. The authors noted that very fine particles of UHMWPE may be indistinguishable from small cement particles due to the resolution limitations of light microscopy. These findings are similar to those described by Goldring *et al.* (1983) and Lennox *et al.* (1987) who analysed tissues from around cemented UHMWPE-metal THRs.

Foreign body giant cells and a large number of intra and extra-cellular particles have also been observed in the PSM (Shanbhag *et al.*, 1994). UHMWPE can be visualised in histological sections using polarised light microscopy and has been observed in both femoral and acetabular tissue sections (Dorr *et al.*, 1990; Schmazlried *et al.*, 1992). There is also evidence that the greater the volume of UHMWPE particles present in the tissues, the greater the number of macrophages in the PSM (Revell *et al.*, 1997; Livermore *et al.*, 1990).

1.5 POLYETHYLENE WEAR PARTICLES *IN VIVO*

Early attempts to examine UHMWPE particles *in vivo* were limited by the resolution of the light microscopy (approximately 1μm). Lee *et al.*, (1992) showed a mean size of polyethylene particles of 2 – 4μm in width by 8 – 13μm in length. Other early studies demonstrated polyethylene particles to be in the size range of 1 – 200μm (Savio *et al.*, 1994). Later studies using higher definition techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed particles in the sub-micrometre size range. Tissue digestion methods using papain concentrated acids or sodium hydroxide, followed by filtration and subsequent examination by SEM revealed particles with a size range of 0.2 – 1.0μm (Campbell *et al.*, 1995; Maloney *et al.*, 1995; Shanbhag *et al.*, 1994).

Maloney *et al.* (1995) examined the wear particles from thirty five membranes obtained during revision hip surgery of UHMWPE-metal THRs after digestion of the soft tissue with papain. The particles were isolated and characterised with the use of light microscopy, SEM and an automated particle analyser. The mean size of the polyethylene particles was 0.5μm, and the metal particles had a mean size of 0.7μm as determined by SEM. The particle analyser revealed a mean particle diameter for polyethylene particles of 0.63μm and a mean of 1.7×10^9 particles per gram of tissue. They suggested that wear debris from prostheses in the tissues around failed femoral components that have been inserted without cement constituted particles that are predominantly less than one micrometer in size.

Margevicius *et al.* (1994) examined wear particles using a technique that involved nitric acid digestion of tissue followed by collection of particles, electronic quantification and parallel morphological and chemical characterisation. The detection limit of the particle counter was approximately 0.58μm. Although a few particles of more than 100μm were detected, the mode of the particle diameter from each sample ranged from approximately 0.58μm to 0.79μm. Particle analysis of tissue adjacent to 20 retrieved total joint prostheses displayed a range of concentration of 0.85 to 141.85 $\times$ 10^9 particles per gram of tissue. These studies indicated that most of the particles in implant membrane were smaller than the resolution of light microscopy and that tissue digestion was necessary for quantification and characterisation. Kobayashi *et al.* (1997) examined UHMWPE particles from three Charnley THRs and showed that the mean particle size was 0.7 $\pm$ 0.05μm and the number of polyethylene particles per gram of tissue was 2.16 $\times$ 10^9.

Tipper *et al.* (2000) quantitatively isolated and characterised all sizes of UHMWPE wear particles from 18 Charnley hip prostheses (mean implant life was 12.88 years, range: 10-19 years). Particles were isolated from the tissues by digestion using 12M potassium hydroxide followed by lipid extraction using chloroform / methanol washes. The resulting supernatants were filtered through 10, 1.0 and 0.1 μm polycarbonate membranes and examined by SEM. The mode of the particle size frequency distribution was 0.1 to 0.5μm for samples from all patients, but particles up to 1mm in length were observed.

These results can be compared to the results of particle analysis following *in vitro* wear of UHMWPE. Matthews *et al.* (1997) generated and characterised UHMWPE particles *in vitro* for use in cell co-culture studies. They used UHMWPE pin articulating against rough stainless steel counterfaces under a constant load. Pyrogen free water was used as a lubricant and the particles were filtered through 10, 1.0 and 0.1μm micropore filters and analysed by SEM. The mean UHMWPE particle size was 0.88 ± 0.13μm.

Heisel *et al.* (2004) investigated the *in vivo* wear of cross linked polyethylene in two groups of patients. Twenty four hips had a conventional polyethylene liner inserted and thirty four had a cross linked polyethylene liner. Linear and volumetric wear rates were measured on radiographs. Compared with conventional polyethylene, cross linked polyethylene showed an 81% reduction in the clinical wear rate. Patients with conventional polyethylene insert showed a linear wear rate of 0.13mm per year compared to a mean linear wear rate of 0.02mm per year with the cross linked polyethylene. Previous hip simulator studies with cross linked polyethylene and conventional polyethylene components have shown wear rates per million cycles of $5mm^3$ and $37mm^3$ respectively (Schmalzried *et al.*, 2000; McKellop *et al.*, 2000). The results of this study are consistent with the reduction of wear with cross linked polyethylene in hip simulator studies.

McCombe and Williams (2004) compared the rate of polyethylene wear between cemented and cementless cups in a prospective randomised trial. In their series,

115 hips (all hips had a 26mm metal femoral head) were studied for differences in rates of wear and osteolysis. The mean clinical follow-up was 8 years and the mean radiological follow-up was 6.5 years. The cementless cups wore at a mean rate of 0.15mm per year (range: 0 – 0.4mm/year) and the cemented cups at 0.07mm per year (range: 0 – 0.22mm/year) and this was statistically significant. The unexpected finding in this study is the wide variation of the rates of wear. Some of this variation may be due to inaccuracies of measurement. A recent review of the literature by Dumbleton *et al* (2002) suggested that osteolysis is infrequent when wear rates are 0.1mm/yr and almost absent when less than 0.05mm/yr. Rates greater than 0.1mm/yr are thought to place the hip at a higher risk of developing osteolysis and eventual loosening.

Kim (2005) in a prospective randomised study compared polyethylene wear in patients who had undergone bilateral total hip replacement with implants that only differed with regards to the material used for the femoral head. Fifty two patients underwent sequential bilateral primary THR with a zirconia head used in one hip and a cobalt-chromium head was used in the other. The mean duration of follow-up was 7. 1 years. Linear wear of the polyethylene liner was measured radiographically. The mean polyethylene wear rate was 0.08mm/yr in association with the zirconia heads and 0.17mm/yr in association with the cobalt-chromium heads. The mean amount and rate of polyethylene wear were significantly lower ($p=0.004$) in hips with a zirconia head than they were in the hips with a cobalt chromium head.

1.6 THE BIOLOGICAL RESPONSE TO POLYETHYLENE WEAR DEBRIS

Macrophages are members of the mononuclear phagocytic system, derived from monocytes in the blood. Their development starts in the bone marrow and passes through several steps (figure 1.2). Macrophages consist of a variety of cell types including Kupffer cells, alveolar macrophages, lymph node macrophages, bone marrow macrophages, pleural and peritoneal macrophages and microglial cells. Originally, it was thought that tissue macrophages were long living cells. However, it has been shown that depending on the type of tissue, their life span ranges between 6 - 16 days (van Furth, 1989).

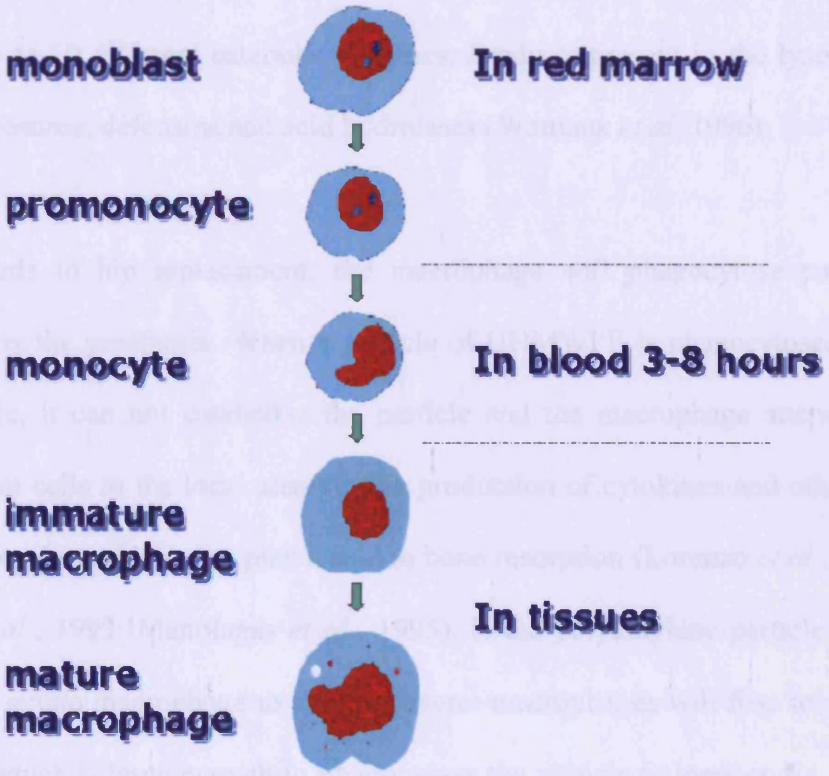


Figure 1.2

Mononuclear Phagocytic System

The primary functions of the macrophage are mainly involved with the inflammatory response and tissue repair. Macrophages participate in both specific immunity via antigen presentation and interleukin 1 (IL-1) production, and non-specific immunity against bacteria, viruses, fungal pathogens, as well as inorganic materials such as silica and carbon (Holian *et al.*, 1997; Lundborg *et al.*, 2001). A major function of the macrophage is non-specific phagocytosis. When a macrophage comes into contact with a foreign material, be it organic such as bacteria or an inorganic particle, it adheres to and engulfs the foreign body and it is internalised into a phagocytic vacuole. The macrophage then attempts to destroy the foreign material by fusing primary lysosomes with the phagocytic vacuole to form a phago-lysosome. The lysosomes have a low pH (4-5) and contain 40 to 50 different catabolic enzymes. Products present in the lysosomes include lysosome, defensins and acid hydrolases (Wattiaux *et al*, 1996).

With regards to hip replacement, the macrophage will phagocytose particles produced by the prosthesis. When a particle of UHMWPE is phagocytosed by a macrophage, it can not catabolise the particle and the macrophage attempts to recruit more cells to the local area via the production of cytokines and other cell signal molecules, which may play a role in bone resorption (Lorenzo *et al.*, 1991; Mundy *et al.*, 1993; Manolagas *et al.*, 1995). If the polyethylene particle is too large for a single macrophage to engulf, several macrophages will fuse to form a giant cell which is large enough to phagocytose the particle (Alpers *et al.*, 1989). In the case where the foreign body can not be degraded by the macrophages or

giant cell, a granuloma will form, which is the body's attempt to isolate the foreign material from the surrounding tissues (Nizegorodcew *et al.*, 1997).

Many studies have reported the presence of cytokines in tissues around revised UHMWPE-metal THRs. Cytokines are soluble, low molecular weight glycoproteins released from a variety of cell types whose properties are the modulation and regulation of other cells. Cytokines in general act on a wider spectrum of target cells compared to hormones. The secretion of cytokines does not necessarily predict the sites at which they exert their biological function. Almost all cytokines are pleitrophic effectors showing multiple biological activities. Cytokines often have overlapping activities and a single cell frequently interacts with multiple cytokines. One of the consequences of this functional overlap is that one factor may frequently functionally replace another factor completely or at least partially compensate for the lack of another factor. Since most cytokines have ubiquitous activities, their significance as normal regulators of homeostasis is often difficult to assess.

The many cell types present in the PSM have been reported to produce a variety of cellular mediators including interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) (Goodman *et al.*, 1998; Yu *et al.*, 1998; Stea *et al.*, 2000). Metalloproteinases, lysosomal enzymes and other substances have also been identified in the PSM and implicated in the resorption of bone associated with implant loosening (Baggliolini *et al.*, 1982;

Mather *et al.*, 1989; Dorr *et al.*, 1990; Horowitz *et al.*, 1993; Chiba *et al.*, 1994; Shanbhag *et al.*, 1994).

It was initially thought that UHMWPE was bioinert. However, many studies have shown that particles of UHMWPE are capable of stimulating macrophages to produce a variety of cytokines (Santavirta *et al.*, 1991; Chiba *et al.*, 1994; Perry *et al.*, 1995; Konttinen *et al.*, 1997). These cytokines may then activate cells whose main functions are remodelling of bone, namely osteoclasts (Suda *et al.*, 1995). By activating these cells in close proximity to the implanted prostheses it is thought that the osteoclasts resorb bone causing loosening of the implant.

Osteoclasts are specialised cells thought to be derived from blood monocytes and are the cells responsible for the removal of bone in normal remodelling processes. They are multinucleated and possess an abundant cytoplasm (Revell *et al*, 1997). They are found attached to the bone surface at sites of active bone resorption and can resorb both the mineral and organic components of bone (Chambers, 1985). Bone resorption occurs via the secretion of acidic products and lysosomal enzymes which breakdown and hydrolyse the collagenous protein and glycosaminoglycans of the bone matrix.

The development and function of osteoclasts involve tightly controlled stages. Enhanced osteoclastogenesis (formation of mature osteoclasts from their precursor stem cells) is a hallmark of bone loss associated with osteolysis and aseptic loosening (Schwarz *et al*, 2000). The differentiation of osteoclasts from

mononuclear phagocyte precursors is known to require the co-operation of osteoblasts and bone stromal cells (Fujikawa *et al*, 1996). There are several important regulators for the formation of osteoclasts that have been identified. One of the identified molecules is receptor activator of NFκ-B ligand (RANKL) and this molecule has been shown to play a central role in the development of osteoclasts (Lacey *et al*, 1998). RANKL was first found on the surface of activated T-cells and later found to be a membrane-bound osteoblast and fibroblast cytokine (Wong *et al*, 1997). RANKL is produced by marrow stromal cells and osteoblasts (Roux and Orcel, 2000). Another important regulator is osteoprotegerin (OPG). OPG is a soluble tumour-necrosis factor-like receptor molecule, which inhibits the formation of osteoclasts by competing for the binding of RANKL to its receptor on preosteoclasts (Lacey *et al*, 1998; Yasuda *et al*, 1998). Activation of OPG suppresses the differentiation of osteoclasts, inhibits their activation and induces apoptosis. Differentiation of osteoclasts begins when RANKL binds to receptor activator of NFκ-B (RANK), which is expressed on the surface of precursors of osteoclasts and mature osteoclasts (Nakagawa *et al*, 1998). RANKL binds to two types of receptors. The first one is RANK, which is expressed in preosteoclasts. The binding of RANK with RANKL triggers a cascade of intracellular events that are essential to completing osteoclast differentiation and activation. RANK is the sole osteoclast receptor for RANKL *in vivo* (Li *et al*, 2000). The second type of RANKL is OPG, which is a decoy receptor that limits the biologic activity of RANKL. Therefore, the balance between OPG and RANKL is essential to regulate bone remodelling, by controlling the activation state of RANK on osteoclasts (Boyle, Simonet and

Lacey, 2003). The role of RANKL in mediating particle-induced osteoclastogenesis and osteolysis has been widely demonstrated (Haynes *et al*, 2001; Clohisy *et al*, 2003; Crotti *et al*, 2004; Clohisy *et al*, 2004; Granchi *et al*, 2004; Granchi *et al*, 2005). Haynes *et* al (2001) were the first to show that RANKL and RANK are associated with wear particle induced periprosthetic bone loss and the formation of osteoclasts from periprosthetic tissues *in vitro*. Mandelin *et al* (2003) showed that RANK and RANKL are involved in wear particle induced formation of osteoclasts in periprosthetic tissue and activated cells in the interface tissue overproduce both RANKL and RANK and they interact without interference by OPG *in vivo*. RANK/RANKL signalling is essential for wear particle induced inflammatory osteoclastogenesis and plays a critical role in the development of aseptic loosening (Ren *et al*, 2006). Osteoclast formation occurs in the presence of M-CSF and RANKL (Lacey *et al*, 1998). However, in the presence of RANKL sufficient M-CSF is produced by cells isolated from periprosthetic tissues to induce osteoclast formation from pseudomembrane derived macrophages (Itonaga *et al*, 2000). Sabokbar *et al* (2003) showed that macrophages derived from the arthroplasty membrane differentiate into osteoclasts in the presence of RANKL alone and that the addition of M-CSF is not required for this to occur. This is because sufficient M-CSF is produced by the activated macrophages derived from periprosthetic tissues to promote osteoclast formation (Neale *et al*, 1999). Sabokbar *et al* (2005) also showed that even fibroblasts in the arthroplasty membrane express RANKL and OPG and that they are capable of supporting osteoclast formation from mononuclear phagocyte precursors by a RANKL-dependent mechanism (Sabokbar *et al*, 2005).

Some cytokines are known to reduce or inhibit bone resorption, whilst others are known to induce it via the stimulation of osteoclast recruitment, maturation and activation. Some authors have suggested that it is the macrophages themselves that directly resorb bone via the release of oxygen radicals and hydrogen peroxide (Amstutz *et al*, 1992; Campbell *et al.*, 1988; Coe *et al.*, 1989).

It has been shown that IL-1β, IL-6, TNF-α, GM-CSF (granulocyte-macrophage colony stimulating factor), PDGF (platelet-derived growth factor) and TGF-α (transforming growth factor-α) are all present in the PSM using immunocytochemistry and *in-situ* hybridisation. All these cytokines may play a role in the process of osteolysis. GM-CSF stimulates the maturation of osteoclast precursors into bone resorbing cells. IL-1, TNF-α and TGF-α have also been shown to induce osteoclast activity (Devlin *et al.*, 1998; Massey *et al.*, 2001).

TNF-α is considered by many to be a major factor in the initiation and development of periprosthetic loosening (Xu *et al.*, 1996). IL-6 also plays a major role in osteolytic lesions in multiple myeloma, a condition that involves bone resorption (Ishikawa *et al.*, 1990). Therefore, both cytokines may have similar effects when produced in the PSM. Other authors such as Ayers *et al.* (1993) and Shanbhag *et al.* (1994) have shown that IL-6 is produced by the PSM and suggested that it may play a role in the process of osteoclast activation and eventually aseptic loosening. IL-1 is also thought to be involved directly in osteoclast activation (Gowen *et al.*, 1983; Dewhirst *et al.*, 1985), or indirectly through its stimulatory effect on the production and activation of other mediators

of osteolysis (Thomson *et al.*, 1986; Dayer *et al.*, 1986). IL-1 is produced by a number of cells such as macrophages, fibroblasts and endothelial cells and has been detected in the PSM (Al Saffar *et al.*, 1994; Chiba *et al.*, 1994; Stea *et al.*, 2000).

Another important enzyme released by macrophages in the inflammatory response is nitric oxide synthase. This chemical mediator is also produced by osteoblasts and osteoclasts and is believed to influence osteoclast activity. Nitric oxide synthase is believed to increase levels of metalloproteinases present in the PSM such as collagenase. All these factors may play a role in the formation of osteolytic lesions seen on radiographs in patients with aseptic loosening (Watkins *et al.*, 1997; Shanbhag *et al.*, 1998).

Nivbrant *et al.* (1999) analysed synovial fluid from 88 hips: 38 had osteoarthritis, 12 were well functioning UHMWPE-metal hip prostheses and 38 were aseptically loose hip prostheses. Levels of TNF-α, IL-1β and IL-6 were measured using Enzyme Linked Immunosorbent Assay (ELISA). Joints with stable or loose prostheses had significantly increased levels of TNF-α compared to those with osteoarthritis. Hips with aseptic loosening had higher levels of IL-1β but not of IL-6 compared with those without an implant. The levels of TNF-α and IL-1β did not differ between stable and loose hip prostheses. These results indicate that cells in the pseudosynovial membrane surrounding hip implants are stimulated to produce pro-inflammatory cytokines in both radiographically stable and loose prostheses.

Granchi *et al.* (1998) investigated whether serum levels of IL-1β, TNF-α, IL-6 and GM-CSF were altered in patients with aseptic loosening of a hip implant, and whether the levels were influenced by the type of material used in the implant. They measured cytokine levels in sera from 35 patients before revision surgery for failed THR and compared them to those in 25 healthy donors. Results from the study showed the serum level of TNF-α did not change. The IL-6 level was not significantly altered, but was higher in patients with titanium prostheses than in those with a chromium cobalt implant and in patients with cemented prostheses. The IL-1β level was found to be higher in those with a titanium cemented prostheses than in the control group. The GM-CSF level was significantly increased in patients compared with healthy donors and it was higher in those with cemented compared to non-cemented implants. Only patients with cementless chromium cobalt implants had levels of GM-CSF similar to those of the control group. These results suggest that several factors can influence the levels of pro-inflammatory cytokines *in vivo* e.g. prosthesis material and whether the implant is cemented or not.

Fiorito *et al.* (2003) also investigated the serum levels of several pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and prostaglandins (PGE_2) as possible early makers of osteolysis in eight patients with periprosthetic osteolysis and ten patients without osteolysis. All patients had a cementless UHMWPE-metal prosthesis implanted. The mean serum levels of IL-1β, IL-6, TNF-α in patients with periprosthetic osteolysis did not differ significantly from those without osteolysis or normal controls. The same authors also investigated the

serum levels of IL-11, an anti-inflammatory cytokine that exerts protective effects on bone resorption. IL-11 serum levels were not detectable in any of the patients, whereas, it was detected within normal range in the control subjects. This provides good evidence that osteolysis is a multi-factorial process (Howell *et al.*, 2000), with some evidence that osteolysis is characterised by local inflammation without the necessity of systemic signs of inflammation.

Stea *et al.* (2000) determined whether there was a correlation between the osteolysis around UHMWPE-metal prostheses and the presence of pro-inflammatory cytokines in tissues at the bone-implant interface. The tissues from twenty nine patients who underwent revision surgery were examined. Immunohistochemistry was performed to detect IL-1α, IL-1β, IL-6 and TNF-α. In tissues collected at the interface membrane, the percentage of cells positive for IL-1, IL-6 and TNF-α increased compared to the tissues collected at the interface from stable implants. The cells occurring in the PSM did not secrete cytokines in quantities that could be related to the severity of wear. Cemented prostheses showed a higher incidence of osteolysis and higher levels of cytokines in the surrounding tissues. Stea *et al.* concluded that TNF-α and to a lesser extent IL-1 and IL-6, were positively related to the severity of osteolysis around the prostheses.

From the above description of cellular reactions leading to osteolysis and the dominant role played by macrophages, it would be expected that the process of phagocytosis of particles may influence the outcome. It has been shown that for

UHMWPE particles, a particular critical size is necessary for optimal activation of the macrophage. Large particle (>10μm) are believed to stimulate macrophages to fuse and form giant cells (von Knoch *et al.*, 2000). Very small particles (<100nm) are thought to be internalised by pinocytosis rather than phagocytosis (Steinman *et al.*, 1983) and may be taken up by any cell type including phagocytes and stromal cells. Some investigators believe that the levels of UHMWPE particles must reach a threshold level in order to initiate the processes involved in osteolysis. This is thought to be approximately 10^{10} particles per gram of tissue (Revell *et al*, 1997). Campbell *et al.* (1996) noted that the total particle concentration in areas of osteolysis surrounding UHMWPE-metal prostheses was approximately ten times higher than in the corresponding capsule tissue, supporting the idea that particle concentration is associated with an osteolytic response by macrophages. Kobayashi *et al.* (1996) also suggested that the incidence of osteolysis depended on the number of polyethylene particles per gram of peri-prosthetic tissue and claimed that osteolysis did not occur until the number of particles per gram of wet interface exceeded 10^9. All these studies displayed that periprosthetic osteolysis might be a dose dependent disease (Kobayashi *et al.*, 1997). However, these studies did not take into account the volume and size of wear debris that is the critical factor in the inflammatory response, and not simply the number of wear particles (Green *et al.*, 2000; Matthews *et al.*, 2000). A large number of small sized particles could theoretically represent a comparatively small volumetric dose if compared to larger particles. Therefore, it would be the concentration of the wear volume that is within the

critical size range of phagocytosed wear debris that would affect the macrophage response.

1.7 THE USE OF CERAMIC MATERIAL IN TOTAL HIP REPLACEMENT

Ceramics combine low density with excellent mechanical properties e.g. high strength and stiffness. They are different from metals in that their bonding is ionic or covalent rather than metallic (Heimke *et al.*, 1974; Nizard *et al.*, 2005). Examples of engineering ceramics are silicon nitride, silicon carbide, alumina and zirconia.

The stiffness of the implant is an important parameter in terms of how it will interact with other components as it transmits the hip load from the femur to the pelvis. Young's Modulus is used to rank the stiffness of biomaterials. Ideally, all neighbouring components should have the same Young's Modulus. When the Young's Modulus is remarkably different, there will be uneven deformation responses and stress concentrations will result. With regards to THR, bone has the lowest values and alumina has the highest values. It can be seen that there is a mismatching between both bone at one extreme and alumina at the other extreme (Hamadouche *et al.*, 2000).

Ceramics do tend to have low toughness and are susceptible to brittle fracture, although new manufacturing processes such as hot isostatic pressing (HIP) have reduced the grain size and increased the density of the alumina, making fractures less likely to occur. Due to ceramics hardness, they have great potential for use in

low friction situations. Alumina shows excellent fracture toughness and bending strength, although not to the same level of zirconia. Alumina has also been shown to be more resistant to wear than zirconia. The concept of an alumina femoral head articulating with an alumina cup has produced the lowest recorded wear rates *in vitro* (Dorlot *et al.*, 1989).

The mechanical properties of zirconia are superior to those recorded for alumina with respect to toughness and bending strength, although it has a hardness less than alumina. Zirconia also has a lower Young's Modulus than alumina, indicating an elastic deformation capability which is not seen in alumina. The increased toughness of zirconia compared to alumina allows zirconia femoral heads to be manufactured in smaller sizes (22mm diameter) compared with the majority of alumina heads (28mm). To date, a large number of zirconia heads articulating with UHMWPE have been implanted in Europe. Whilst the alumina-alumina combination has been shown to produce the lowest recorded rates of wear *in vitro*, the zirconia on zirconia combination does not show such favourable results with high wear *in vitro* (Hummer *et al.*, 1995).

Zirconia was introduced as an alternative to alumina ceramic. Femoral heads made from zirconia are considered to be less likely to fracture and to have a lower wear rate against UHMWPE than femoral heads made from stainless steel or cobalt chrome (Derbyshire *et al*, 1994; Cales *et al.*, 2000). Zirconia's ability to withstand fracture is four times greater than that of alumina (Masonis *et al.*, 2004). Hernigou *et al* (2003) analysed UHMWPE wear in hips with a femoral

head made from zirconia. They calculated the annual wear at five years to be 0.04 mm but this increased to 0.41 mm at 12 years. von Schewelov *et al.* (2005) investigated the wear characteristics of four different total hip joint articulations in 114 patients. In their study, they were unable to verify the reduction in wear which has been reported in *in vitro* studies for femoral heads made from zirconia rather than stainless steel. In contrast, the total and annual wear using zirconia was actually increased by approximately 50%. von Schewelov *et al.* concluded that zirconia femoral heads should not be used in THR.

1.8 HISTORY OF CERAMIC TOTAL HIP PROSTHESES

The use of ceramic materials for orthopaedic implants in humans gained recognition in 1933 (Rock, 1933). However, it was Sandhaus in 1965 who proposed alumina for clinical usage (Sandhaus, 1965). This prompted intensive investigation into the use of ceramic material for artificial joints by Hentrich *et al.* (1971), Heimke *et al.* (1974) and Griss *et al.* (1973).

Boutin introduced alumina ceramics in 1970 as an alternative to UHMWPE in total hip replacement. He experimented with both cemented and non-cemented components (Boutin & Blanquaert, 1981). Boutin was concerned that the wear rates of polyethylene might be problematic in the future. He thought that by using ceramic components, the wear rate would be reduced. Boutin examined 1261 cases of both cemented and non-cemented alumina ceramic on ceramic THRs in 1983, 13 years after the first ceramic on ceramic THR. Only six femoral head and four socket fractures were seen, along with three cement fractures and seven cases

of visible wear. Although these results were promising, early reports suggested that both femoral and acetabular alumina components could break under high loads and that the combination of a very hard material such as alumina with the relatively soft structure of cancellous bone might prove problematic in not being supportive (Griss & Heimke, 1981; Plitz & Hoss, 1982). Several reports have been reported regarding the doubt on the overall safety of ceramic on ceramic implants (Higuchi *et al.*, 1995; Michaud & Rashad, 1995; Kricker & Schatzker, 1995).

Wallbridge *et al* (1983) thought that implanting ceramic prostheses into young, active patients might increase the life expectancy of the implant. Sedel *et al.* (1990) reported a 94.6% survival rate at ten years from cemented alumina on alumina THRs in patients under 50 years of age. The results for older patients were not as good and was thought that the decreased rigidity of the acetabulum and the tendency for elderly bone to be more brittle was the reason for lower success. However, there are still some limitations of ceramics. The femoral head size must not be too small or there is an increased risk of fracture. The articular surfaces must be accurately designed with a clearance of 15-40μm between the two alumina components (Nevelos *et al*, 1999).

Huo *et al.* (1996) reviewed the results of 112 ceramic on ceramic Mittelmeier THRs implanted without cement. Ninety three of the replaced hips were followed for a minimum period of 60 months, with an average of 108 months. Five and a half percent of the hips were revised due to loosening. Of the remaining hips,

87.5% were rated clinically satisfactory. However, overall mechanical failure rates were 21.5% for the acetabular cup and 22.6% for the femoral stem. It was noted that no femoral or acetabular osteolysis was observed and failure was attributed to mechanical problems.

Examples of the ceramic on ceramic artificial hip joint implants used were the Mittelmeier and Ceraver-Osteal prostheses. Both these implants are constructed from alumina. Miller *et al.* (1986) were one of the first groups to report on the clinical experience of the Mittelmeier prosthesis outside Germany. Of the 231 replaced hips, five revisions were reported after a short follow-up period of 1-3 years. O'Leary *et al.* (1988) reported on a single centre set of Mittelmeier hips. Of 69 (44 primary) hips with a mean follow-up of 39 months, 19 hips required revision surgery with the main reason for failure being aseptic loosening in all but one. 10 of these revisions were part of the primary group, which gave a 22.7% failure rate. Loosening in the majority of the replaced hips was attributed to an abduction angle greater than 50^{o} and inadequate canal filling due to undersized femoral stems.

Refoir *et al.* (1997) reported wear debris production by the Mittelmeier prosthesis. They found the mean ceramic wear rates of between $1.9\mu m^3$ to $3.8\mu m^3$ per one million cycle of hip simulator testing. When examining explanted prostheses, Mittelmeier *et al* reported mean linear wear rates of 2.6μm per year for the acetabulum and 5.4μm per year for the femoral head (Mittelmeier *et al.*, 1992).

Mahony *et al* (1990) reported a combined linear wear of 19μm per year for both acetabulum and femoral head. Jazrawi *et al.* (1999) performed clinical and radiographic analysis on 58 patients (mean age: 45.2 years) at a minimum of 10 year follow-up (mean: 12.7 years) after THR using an Autophor ceramic on ceramic THR. The mean wear rate at final follow-up was 0.016mm per year. There were no cases of periprosthetic osteolysis in the acetabulum or femur. For the unrevised components, there were three (5%) cases of protrusion acetabuli and four (7%) cases of acetabular component loosening. Regarding the femoral component, 83% had greater than 2mm implant-bone radiolucencies in more than five Gruen zones as a result of gross motion of the stem. Despite radiographic evidence of implant loosening, this bearing articulation functioned well for more than 12 years with remarkably low wear.

The clinical status of 82 Mittelmeier ceramic on ceramic (mark II) cementless total hip prostheses (Autophor, Osteo AG) implanted between 1978 and 1984 were analysed by Garcia-Cimbrelo *et al.* (1996). One or both components of 12 hips were revised (16% at 16 years in the survival study). 11 acetabular components and seven stems were revised, with three of the acetabuli fractured. Radiographic cup loosening was observed in 53% of hips at 16 years with most having a complete radiolucent line of 2mm around the cup, whereas radiographic stem loosening appeared in 15% of hips after the same time period. Retrieved tissue from the revised hips was studied histologically. Fibrous tissue was present in 64% of stems. Significant radiographic acetabular wear was not observed in

any hip, although intracellular ceramic wear debris was seen in the retrieved tissues.

Sedel published data in 1994 of the results of Ceraver-Osteal alumina on alumina hip replacement in patients younger than 50 years old. From 1977 to 1990, 131 THRs were performed on 113 patients with a median age of 41 years. 106 sockets were cemented with 25 uncemented. The mean follow-up period was 5 years, with 32 hips followed for more than 10 years. Revision arthroplasty was considered as failure and an endpoint. Survivorship analysis showed a 97.5% rate of survival at five years, 89.4% rate at 10 years and 86.2% at 11 years. Nine revisions were performed: one periprosthetic fracture after three years, one bipolar loosening, one femoral cystic lesion formation and six were revised for acetabular loosening, all from the cemented group (Sedel *et al.*, 1994). These results suggested an excellent survival rate for ceramic on ceramic THRs in young active patients.

Bohler *et al.* (2000) reviewed alumina ceramic sockets implanted between 1976 and 1979 (Group 1) and titanium sockets with alumina liners (Group 2) implanted between 1990 and 1995. Both cementless sockets articulated with alumina ceramic femoral heads. Clinical follow-up of patients in group 1 showed a total failure rate of 19.6% after 5 to 20 years. Radiographic analysis of eight stable sockets showed migration of 0.2mm to 2.89mm, but in four sockets at risk for late aseptic loosening and failure after an average follow-up of 12.5 years as much as 13.4mm of migration was seen. Evaluation of the periprosthetic tissues revealed pseudosynovial membranes as thick as 1mm with fine birefringent wear particles

within macrophages around two stable retrieved sockets visible in histological sections. The membranes around four loose sockets were 6 to 10mm thick and heavily loaded with larger alumina wear particles. A mean annual linear wear of 38.8μm was calculated for the bearings in group 1 and 26.94μm for bearings in group 2. These results supported the good tribological and biological properties of alumina ceramic bearings for THR.

Hyder *et al.* (1996) examined 26 Mittelmeier cementless ceramic hip replacements performed in 22 patients with an average age of 24 years (range: 17 to 30 years) at the time of operation. 21 patients (25 hips) were reviewed with an average follow-up period of 6.5 years (range: 4 to 13 years). Two hips had to undergo revision of the ceramic acetabular components, one hip was explored due to wound discharge. Harris hip scores were good to excellent in 64%, fair in 16% and poor in 20% of cases. Apart from two sockets revised, progressive radiolucent lines were seen in five cups (20%) and significant vertical migration was present in nine cups (36%) with an average migration of 1.3mm per year. In total, acetabular changes were observed in 11 cases (44%). Femoral stem subsidence of between 3 and 6mm was seen in five cases (20%). The total number of hips showing loosening of one or both components was five (20%). Overall radiographic changes (migration and loosening) in one or both components were present in 13 hips (54%).

Nevelos *et al.* (2001) compared and quantified the modes of failure and patterns of wear of 11 Mittelmeier and 11 Ceraver-Osteal retrieved alumina on alumina

hip prostheses with reference to the corresponding clinical and radiological histories. Macroscopic wear, surface roughness and mechanism of wear at the sub-micrometre level were determined. The components were classified into one of three categories of wear: low (no visible/measurable wear), stripe (elliptical wear stripe on the heads and larger worn areas on the cups) and severe (macroscopic wear, large volumes of material lost). Overall, the volumetric wear of the alumina on alumina prostheses was substantially less than the metal on polyethylene combination, less than $1mm^3$ per year compared to $48\text{-}80mm^3$ per year respectively. These results suggested that correctly implanted alumina on alumina bearings represents a superior choice compared to metal on UHMWPE as the bearing surfaces in THR.

Bizot *et al.* (2004) reviewed 62 patients under the age of 55 years (mean age: 46 years) who had alumina on alumina hip replacements (Ceraver-Osteal) between 1990 and 1992. The prosthesis involved a cemented titanium stem, 32mm alumina head and a press fit socket with an alumina insert. The nine year survival rate for aseptic loosening was 98.4%. The outcome in the surviving 50 patients with a minimum of five year follow-up was excellent in 47 hips (82.5%), very good in eight (14%), good in one and fair in one. A thin lucent line was present in 38% of the sockets. One stem had femoral osteolysis but there was no detectable component migration nor acetabular osteolysis. Overall, this hybrid alumina on alumina prostheses gave satisfactory results in young active patients.

Tanaka *et al.* (2003) examined the behaviour of alumina ceramic heads in 156 cemented THR at a minimum follow-up of eight years. The hips were divided into three groups for measurement of wear based on the size of femoral heads; 22, 26 and 28 mm. All alumina heads articulated with all-polyethylene acetabular components. The polyethylene wear was measured radiologically using a computer aided technique. The linear wear rate of polyethylene for the 28 mm heads was high (0.156 mm/yr) compared for the 22 and 26 mm heads where the wear rates were relatively low (0.090 and 0.098mm/yr respectively). Four hips were revised within eight years of index operation, three for aseptic loosening and one for infection. The surface roughness of the three retrieved femoral heads showed maintenance of an excellent finish of the alumina. This study was able to show a reduced wear rate with the use of alumina heads and have excellent surface quality.

Yoo *et al.* (2005) reported the results of an alumina on alumina THR with regards to wear, osteolysis and fracture of the ceramic after a minimum duration of follow-up of five years. The series consisted of 100 consecutive primary THR from a single surgeon in patients who were sixty five years of age or younger. A titanium alloy backed socket and cementless stem titanium alloy were used. No implant was loose radiographically and no implant was revised. Periprosthetic osteolysis was not observed in any hip. A fracture of the alumina femoral head and a peripheral chip fracture of the alumina insert occurred in one hip following a motor-vehicle accident. The findings of this study suggest that alumina on

alumina THR are associated with excellent clinical results and implant stability at five years. In addition, no wear or osteolysis was observed.

1.9 ALUMINA WEAR PARTICLES *IN VITRO*

In vitro simulation of the wear of alumina ceramic hip replacements have been carried out using simple configuration wear tests and hip simulator studies. Early studies used water as the lubricant when testing the alumina on alumina coupling and found high wear rates (Wallbridge *et al.*, 1983; Lancaster *et al.*, 1992). Wallbridge *et al.* (1983) used simple pin on disc and pin on plate configurations, run both dry and distilled water as the lubricant. Results from their studies estimated that the alumina on alumina would wear at a rate of about half of the rate of UHMWPE on metal and only slightly less than alumina on UHMWPE. However, these simulator configurations were not clinically relevant.

Studies using hip simulators have provided more physiological testing. Hip simulators have been validated for the testing of UHMWPE on metal because of the large numbers of retrieved components available for direct comparison of the wear rates and mechanisms. Several different simulators have been used to test alumina on alumina hip prostheses. Oonishi *et al.* (1999) reported on a 10 million cycle alumina on alumina simulator study conducted using a non-anatomical simulator with bovine serum lubricant. This study was designed to investigate the effect of diametrical clearance on the wear rate. They tested three different clearances (20-30μm, 60-70μm and 90-100μm) and found no significant difference in wear rates and the overall wear was minimal with acetabular cup

wear rates less than 0.01mm^3 per million cycles. Clarke *et al.* (2000) studied the wear of the alumina on alumina Kyocera prosthesis in a physiological servo-hydraulic hip simulator with bovine serum used for lubrication. The alumina on alumina combination wore at the steady rate of 0.004mm^3.10^6 cycles over 10 × 10^6 cycles. Taylor (1999) reported on a simulator study that compared the wear of UHMWPE on metal and alumina on alumina hip joints. The alumina femoral head had a diameter of 28mm. The components were held in the anatomical position with 30% (v/v) bovine serum as the lubricant. The average wear rate of the alumina on alumina bearings was 0.025mm^3 per million cycles compared to 34mm^3 per million cycles for the UHMWPE on metal bearings.

Hot Isostatic Pressing (HIP) is a process that subjects a component (metal or ceramic powder) to elevated temperatures and pressures to eliminate internal micro-shrinkage. The reduced porosity of HIPed materials enables improved mechanical properties. This process eliminates internal voids and creates uniform micro-structure. Refior *et al.* (1997) carried out a simulator study of alumina on alumina hips made from HIPed and non-HIPed material in a simulator which had three axes of motion and used a single axis twin peak loading cycle. The study was conducted in water in order to produce more severe wear conditions. The average wear rate for the non-HIPed alumina was 1.5mg per million cycles (0.4mm^3 per million cycles). The HIPed material had a lower wear rate of 0.2mm^3 per million cycles.

Tipper *et al.* (2000) investigated the wear rates of the alumina on alumina coupling in a hip joint simulator. The femoral heads had a diameter of 28mm and three hips were tested. A physiological hip joint simulator was used with the cup in the superior position to the head and inclined in the anatomical position. A single axis twin peak loading curve was applied through the vertical axis of the cup. Two directions of motion were applied, flexion extension and internal external rotation. Wear volumes were determined every million to five million cycles. Tests were carried out in 25% (v/v) bovine serum. The steady state of wear was $0.05mm^3$ per million cycles.

The wear rates for the alumina on alumina coupling *in vivo* were far greater than those produced in physiological hip simulators. The hip simulators were also not reproducing the wear patterns observed in retrieval studies of non HIPed alumina on alumina prostheses (Nevelos *et al.*, 2000), namely a wear "stripe" on the femoral heads and some wear on the rim of the acetabular cups. Nevelos *et al.* (2000) therefore attempted to establish the *in vivo* wear patterns of HIPed alumina prostheses with reference to the wear patterns of non-HIPed alumina seen in the retrieval study. Fluoroscopy studies had shown micro-separation could occur with any total hip prosthesis and could be a factor in the wear of ceramic on ceramic prostheses. The small clearances of the head and socket (typical radial clearances are of the order of 40μm) mean that it is possible that the femoral head will translate inferiorly and laterally. These displacements could typically be less than 1mm for a well positioned prosthesis. During heel strike, the head will translate superiorly and contact the acetabular before re-locating. This rim contact will

occur under high stresses and may initiate surface damage and hence accelerate wear. Nevelos *et al.* (2001) reproduced this micro-separation in a physiological hip joint simulator. Wear volumes for the coupling were 1.7mm^3 after 8×10^5 cycles for the non HIPed alumina and 1.2mm^3 after 8×10^5 cycles for the HIPed alumina. They also reproduced the stripe wear seen on alumina femoral heads after revision.

Tipper *et al.* (2002) investigated the wear patterns of alumina using a hip simulator with micro-separation of the prosthesis components during the swing phase. Three 28mm femoral heads were tested using 25% bovine serum lubricant. In order to achieve micro-separation, they reduced the vertical load and a force was applied in the medio-lateral direction using a spring. The initial displacement in this direction was 800μm but decreased to 200μm after 100,000 cycles. Wear particles produced after micro-separation were 1.24mm^3 per million cycles for HIPed components and 1.74mm^3 per million cycles for the non-HIPed components. The wear stripe often seen clinically on the femoral head was reproduced on both types of prostheses. They were able to show that when micro-separation was introduced into the simulation, the wear rates increased. In addition, the introduction of micro-separation produced larger wear particles (0.02 to 1μm). They concluded that clinically relevant wear rates, wear patterns and distribution of wear particles were produced by the introduction of micro-separation during the swing phase of their *in vitro* hip joint simulation.

1.10 HISTOLOGY ANALYSIS OF TISSUES FROM AROUND CERAMIC TOTAL HIP REPLACEMENT

It is possible to histologically visualise ceramic wear debris in tissues around ceramic on ceramic THRs. The accumulation of ceramic wear debris has been shown to induce an inflammatory reaction, one that is less intense than the reaction seen with polyethylene debris (Boutin *et al.*, 1988). Boutin *et al.* demonstrated that as the amount of alumina wear debris increased in periprosthetic tissue, the inflammatory response was more pronounced. They examined tissue sections from loosened alumina on alumina components and reported the presence of numerous alumina particles less than 5μm in diameter, often located within macrophages and associated with a mild inflammatory reaction. Periprosthetic tissues from well fixed uncemented prostheses were investigated by Kummer *et al.* (1990). They observed an abundance of alumina particles with a mean size of 5μm in the tissue, as well as a mild inflammatory reaction in association with the alumina debris. Henssge *et al.* (1994) reported on the ceramic debris in periprosthetic tissues from cemented alumina on alumina prostheses that had been revised for loosening. They observed sharp edged polygonal yellow-brown particles of up to 5μm in diameter and smaller, granular debris within macrophages in all tissue specimens. Particles within the soft tissue from the interface membrane of the acetabular components of Cerevar-Osteal prostheses were isolated by Lerouge *et al.* (1996). The analysis of these particles by SEM revealed a mean size of 0.44 ± 0.25μm. Tissues from three Mittelmeier THRs failed because of aseptic loosening were analysed by Yoon *et al.* (1998). They observed abundant ceramic particles with a mean size of 0.71μm (range:

0.13 to 7.21μm). Bohler *et al.* (2000) compared wear particles from two different types of alumina on alumina THR. They reported a mean size of alumina particle of 0.608μm (SD - 0.06) in one group and 0.586μm (SD – 0.006) in the other group as determined by SEM.

Hatton *et al.* (2002) isolated ceramic wear particles from the periprosthetic tissues from around Mittelmeier ceramic on ceramic hip prostheses. Characterisation of the wear particles by TEM and SEM revealed bi-modal size distribution. SEM revealed particles in the 0.05 – 3.2μm size range, whereas, TEM revealed particles in the 5 – 90nm size range. This was the first time that nanometre sized ceramic wear particles were shown to be present in periprosthetic tissues. These wear particles sizes were associated with roughened areas of the bearing components.

Kummer *et al.* (1990) analysed tissues removed from well fixed ceramic on ceramic components. The tissues which contained numerous alumina particles (mean diameter 5μm) exhibited a mild inflammatory reaction. In addition, tissue sample analysis from ceramic bearing components inserted with bone cement exhibited additional particulate debris, including PMMA and cement.

1.11 THE BIOLOGICAL RESPONSE TO ALUMINA WEAR DEBRIS

It is known that ceramics are one of the least biologically reactive materials. A bioinert material is a material that should not induce a tissue response after surgical implantation. The receiving host should ignore the material and the tissue surrounding the implant should display normal structure and activity (Christel *et al*, 1988). However, ceramic prostheses implanted into the body are eventually surrounded by a fibrous capsule indicating that they are not bioinert. Ceramic wear debris is generally considered to be insoluble due to it being in its highest oxidation state.

There is a limited amount of data in the literature on the biological responses to ceramic wear debris. The biological reaction to wear debris has been reported to be governed by the quantity present in the tissues surrounding the joint. The volume of ceramic wear debris produced is many times less than the amount produced in a UHMWPE hip joint. Several authors have suggested that both alumina and zirconia may play a role in the process of aseptic loosening of ceramic on ceramic THRs. Shih *et al.* (1994) showed femoral osteolysis in eight out of 134 cementless ceramic on ceramic THRs performed during an average nine year period. The lesions were usually located in the deeper periprosthetic area.

Wirganowicz *et al* (1997) reported massive femoral osteolysis distal to a Mittelmeier cementless femoral ceramic implant. The femoral components and

acetabulum showed signs of visible wear and tissue removed from the femoral medullary canal contained extensive macrophages and levels of particulate wear debris. Analysis of the debris by energy dispersive x-ray (EDX) showed alumina to be 10 times more prevalent that either cobalt or chromium. EDX is a chemical micro-analysis technique that utilises X-rays that are emitted from a sample during bombardment by an electron beam to characterise the composition of an analysed sample. This extensive osteolysis may have indicated a possible role played by alumina debris in the process of aseptic loosening.

Lerouge *et al.* (1996) suggested that ceramic wear debris might play a part in the process of aseptic loosening in ceramic on ceramic THRs. Systematic characterisation of the *in vivo* wear debris from 15 cases of ceramic on ceramic THRs was performed using semi-quantitative histological analysis of haematoxylin and eosin (H&E) stained periprosthetic pseudosynovial membranes and an evaluation of the isolated ceramic debris was carried out using EDX, SEM and image analysis. The results showed that the most predominant wear particles were zirconium dioxide [ZrO_2] (76%) with alumina debris present in the smallest proportion (12%). Zirconium ceramic was used as the opacifying agent in the cement for their fixation of the prostheses. A histiocytic foreign body reaction was noticed to the ZrO_2 debris in tissue sections suggesting that these particles may have played an important role in aseptic loosening of the ceramic on ceramic THRs analysed in this study. Lerouge *et al.* (1997) then semi-quantified histology of pseudomembranes from 12 loose cemented ceramic on ceramic and 18 metal on polyethylene THRs. Their study suggested that aseptic loosening of the

ceramic cups was not due to a response to debris generated at the articular interface, but to mechanical factors which led to fragmentation of the cement.

Yoon *et al.* (1998) reviewed 103 non cemented Mittelmeier ceramic on ceramic prostheses. 23 femoral and 49 acetabular components had radiological signs of osteolysis at a mean implantation time of 92 months. The osteolysis associated with the femoral component was reported to be linear in 12 and scalloping in 11. The acetabular osteolysis was linear and not focal. Ten of the patients were revised for loosening of the acetabular component. The interface tissue was reported to be a vascular fibrous connective tissue rich in macrophages containing electron dense material within phagosomes. Abundant ceramic wear particles were observed and they concluded that ceramic wear particles could stimulate a foreign body reaction leading to periprosthetic osteolysis.

It is known that with polyethylene wear debris, particle size is critical in the stimulation of cytokine production by macrophages, which could eventually lead to periprosthetic loosening. Green *et al.* (1998) reported that a particle size of approximately three microns was the most effective stimulator of mouse peritoneal macrophage to produce various cytokines including TNF-α, IL-1, IL-6 and PGE_2. Nagase *et al.* (1995) assessed the role of alumina crystal size in the biological response to alumina debris. The work involved the use of different sized alumina particles to test for the generation of reactive oxygen metabolites in human polymorphonuclear leucocytes (PMN) or mononuclear leukocytes. On an equal weight, alumina particles of different sizes induced PMN in the following

order of magnitude: 3.2μm > 7.5μm, 0.8μm > 0.6μm > 28μm, 68μm. On the basis of identical surface areas, alumina particles of different sizes elicited PMN in the following order of magnitude: 3.2μm > 7.5μm, 0.8μm > 0.6μm, 28μm, 68μm. It was suggested that the reason for the 3.2μm sized crystals having greater effect was that this particular size may interact with the leukocytes more efficiently by attaching to specific surface receptors or adhesion molecules that are of an optimal size for the crystal. These results suggested that the critical size estimation by Green *et al.* (1998) may also apply to alumina crystals although further investigation needs to be carried out into their ability to induce cytokine production.

Using a rabbit model, Kubo *et al.* showed a marked histiocytic response around particles of UHMWPE (mean diameter 11μm) and cobalt chrome [CoCr] (mean diameter 3.9μm). They noted a less intense histiocytic response surrounding particles of alumina ceramic (mean diameter 3.9μm) and titanium (Ti) (mean diameter 3.9μm). Catelas *et al.* (1998) investigated the biological effects of ceramic and high density polyethylene particles by analysing their ability to stimulate the release of TNF-α from the mouse macrophage cell line, J774. They tested alumina particles of 0.6, 1.3, 2.4 and 4.5μm, zirconia particles of 0.6μm and high density polyethylene particles of 4.5μm at 5-2500 particles per cell over a 24 hour period. However, they did not state that the particles were endotoxin free. For the alumina particles, they found a direct relationship between the size of the particles and TNF-α production, with no difference between the alumina and zirconia particles of the same size. High density polyethylene released higher

levels of TNF-α compared with alumina at the same size and dose. They concluded that the macrophage response was most probably dependent on the volume of particles phagocytosed and that alumina had a clinical advantage over polyethylene.

Hatton *et al.* (2003) investigated the effects of clinically relevant alumina wear particles on TNF-α production by human peripheral mononuclear phagocytes. Particles were generated in a hip simulator under micro-separation conditions. The particles showed a bimodal size distribution with nanometre (5-20nm) and larger particles (0.2-10μm). The particles were cultured with cells at different particle volume to cell number ratios. All cells produced significantly elevated levels of TNF-α when stimulated with $500\mu m^3$ of micro-separation wear particles cell. Only half the cells secreted significantly elevated levels of TNF-α when stimulated with $100\mu m^3$ of the micro-separation wear particles. They concluded that alumina wear particles generated under micro-separation conditions are capable of inducing cytokine production by macrophages. However, a high volumetric concentration of the particles is required to generate this response which is unlikely to occur *in vivo*.

Germain *et al.* (2003) compared the cytotoxicity of clinically relevant cobalt-chrome (CoCr) and alumina wear particles on the viability of histiocytes and fibroblasts. CoCr wear particles were generated using a pin on plate tribometer. Clinically relevant alumina wear particles were generated using a hip simulator with micro-separation motion. CoCr particles cultured with both histiocytes and

fibroblasts resulted in a significant reduction in adenosine triphosphate (ATP) levels at particle volumes of 50 and 5μm^3 per cell, demonstrating the cytotoxicity of these particles. However, alumina were shown to be less cytotoxic than the CoCr particles.

1.12 BEARING SURFACES OF TOTAL HIP REPLACEMENT

Throughout the history of THR, the metal on UHMWPE prosthesis has provided an excellent choice for both surgeons and patients providing many years of pain-free movement and relief. The Charnley prosthesis is still commonly used today, although alternate bearing materials such as ceramic on ceramic and metal on metal do offer the alternative for lower wear volumes of particulate wear debris, which is believed to be the primary cause of osteolysis and aseptic loosening. Osteolysis is an ever increasing problem in THR and long term clinical success will depend on the use of quality, low wearing materials and good surgical technique.

The re-emergence of ceramic bearing surfaces for total hip replacement after the clinical success of UHMWPE on metal bearings has been primarily due to the association between polyethylene wear debris and periprosthetic osteolysis. Periprosthetic osteolysis is often seen in the younger, more active and higher demand patients. Ceramic bearings with lower wear rates, can potentially improve the longevity of the implant for the higher demand patient by decreasing particulate debris generation and the resulting osteolysis. Although initial prospects are encouraging, more studies are needed on the characteristics of the

wear particles generated *in vivo* before predictions concerning the potential long term success of the ceramic on ceramic bearings. It is essential that pre-clinical testing of any new ceramic materials for joint replacement must include an analysis of the wear particle characteristics and their biological reactivity.

Resurfacing arthroplasty of the hip using a metal on metal prosthesis with a cemented femoral component and uncemented acetabular socket has been advocated for younger patients with high demand. The concept of resurfacing the hip is not new and Sir John Charnley attempted the procedure. This new prosthesis design is intended to overcome the problems of aseptic loosening due to polyethylene wear particles. The results of the Birmingham hip resurfacing arthroplasty have recently been published by one of pioneers of this implant whom report a cumulative survival rate of 99.8% at four years (Daniel *et al*, 2004). Another independent prospective study of 230 primary Birmingham hip resurfacing arthroplasty has recently been published by Back *et al* (2005). At a mean follow-up of three years, survivorship was 99.14% with revision in one patient for a loose acetabular component and one death from unrelated causes. One patient developed a fracture of the femoral neck at six weeks. Most patients (97%) in this series considered the outcome to be good or excellent. These two studies have shown very satisfactory outcomes of the Birmingham hip resurfacing arthroplasty but further independent long term studies are still required.

1.13 AIMS OF INVESTIGATION

The aims of the study were to test the hypothesis that clinically relevant alumina ceramic particles implanted in radiolabelled murine femora will establish a small animal model of wear debris induced osteolysis that can be used to screen potential pharmaceutical agents. A large part of this study concentrates on exploring the possibility of developing a simple and cheap *in vivo* animal model of osteolysis.

Specific objectives:

- To quantify osteolysis caused by alumina ceramic particles that have been implanted into murine femora over different time intervals.
- To demonstrate N-telopeptide and deoxypyridinoline are generated during bone resorption in the model and utilise these breakdown products as osteolysis makers.
- To investigate the presence and quantification of matrix metalloproteinase-9 in the model of wear debris induced osteolysis over different time intervals.

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2 GENERAL MATERIALS AND METHODS

This chapter contains the materials and methods that were utilised commonly throughout the study. Specific techniques involved are described in the individual chapters.

2.1 MATERIALS

2.1.1 Home Office Licences

Both project and personal licences were obtained from the Home Office in order to carry out surgical experiments on laboratory animals. All surgical procedures took place at the Division of Biomedical Services (DBS) at the University of Leicester. National guidelines for the care and use of laboratory animals were observed.

Project Licence Number: PPL 40/2631

Personal Licence Number: PIL 40/7467

2.1.2 Animals

All animals were bred at the DBS. C57/Bl6 female mice were time mated with CBA male mice to produce the B6CBF^{1} progeny, also known as the F^{1} hybrid. Period of gestation was on average between 20-22 days. The pregnant female mice were injected subcutaneously with radioisotope ^{45}Calcium Chloride on day 14 of gestation. Offspring were kept with their mothers for three weeks. Following weaning of the offspring, females and male offspring were then

separated into different cages. Offspring of the same litter were identified by ear codes.

2.1.3 Chemicals

Sterile pyrogen-free water was supplied by Baxter Healthcare Ltd., Berkshire, UK. Hydrochloric acid was purchased from Merck Ltd., Leicestershire, UK. E-Toxaclean® detergent was supplied by Sigma-Aldrich Company Ltd., Dorset, UK. Phosphate buffered saline (PBS) tablets were obtained from Oxoid Ltd., Hertfordshire, UK.

2.1.4 Consumables

Twenty five millimetre polycarbonate hydrophilic filters (Cyclopore™ track etched membrane) were supplied by Whatman International Ltd., Maidstone, UK in 10, 1, 0.1 μm pore sizes. Powder free Ultrasafe® latex examination gloves were produced by Ultrawin SDN.BHD, Perak, Malaysia and supplied by Fisher Scientific, Leicestershire, UK.

2.1.5 Enzyme Linked Immunosorbent Assay (ELISA) kits

N-Telopeptide (NTx) and deoxypyridinolone (DPD) were assayed using commercial ELISA kits purchased from Quidel Diagnostics, Oxfordshire, UK.

2.1.6 Endotoxin assay

The turbidometric endotoxin assay, Pyrotell-T®, was supplied by Associates of Cape Cod International, Liverpool, UK. The kit consisted of a 32 well

spectrophotometer, limulus amoebocyte lysate (LAL) produced in the horseshoe crab *Limulus polyphemus*, control standard endotoxin (CSE) and pyrogen free water.

2.1.7 Equipment

The sonicating bath used for particle sonication and separation was manufactured by Sonics and Materials, Connecticut, USA. A six decimal balance (Sartorius micro-balance 2405) was used for the weight determination and was manufactured by Sartorius Werke, Göttingen, Germany. The ion stream (Stat-Attack) was manufactured by Amersham International Ltd., UK. Scanning Electron Microscopy was performed on a Hitachi S3000H emission microscope (Hitachi, Tokyo, Japan). Image analysis was performed using Image Pro Plus 3.0 software (Media Cybernetics, USA). The centrifuge and plate spinner were manufactured by M.S.E Scientific Instruments, Crawley, UK. The LAL-500 Automatic Endotoxin Detection System and Pyros® software were manufactured by Associates of Cape Cod International, Liverpool, UK.

2.1.8 Particles

Polyethylene and alumina ceramic powder where supplied by the Department of Microbiology, University of Leeds.

2.1.8.1 Polyethylene particles

Polyethylene powder was obtained from Ceridust, Germany.

2.1.8.2 Alumina ceramic particles

Alumina ceramic powder was obtained from Ceramtec, Plochingen, Germany.

2.1.9 Plasticware

Endotoxin free pipette tips and serological pipettes were purchased from Associates of Cape Cod International, Liverpool, UK. Pyrogen free serological pipettes were manufactured by Corning Costar UK Ltd., Buckinghamshire, UK. Petri dishes, bijous and universal containers were supplied by Bibby Sterilin, Staffordshire, UK.

2.1.10 Radioisotope

The ^{45}Calcium Chloride ($^{45}CaCl_2$) radioisotope was purchased from Amersham Life Sciences Ltd., Oxfordshire, UK. $^{45}CaCl_2$ were supplied in 0.5ml containers with a radioactivity of 37MBq.

2.1.11 Syringes and needles

Microlitre syringes and needles were purchased from VWR International Ltd., Leicestershire, UK.

2.1.12 Syringe-tip filters

25mm syringe-tip filters were purchased from Fisher Scientific, Leicestershire, UK.

2.2 METHODS

2.2.1 Sterilisation

All glassware and surgical equipment used throughout the study was kept solely for use in this study to prevent cross-contamination. Glassware was washed in 0.15% (w/v) Neutracon (Fisher Scientific) followed by 3 rinses in tap water and 3 rinses using distilled water. Sterilisation was achieved by dry heat at 180°C for 5 hours. Plugged Pasteur pipettes were sterilised in canisters by dry heat at 180°C for five hours. Surgical instruments were autoclaved at 121°C for 20 minutes in an autoclave (British Steriliser, British Steriliser Co. Ltd., London, UK). Heat labile solutions were filter sterilised through 0.2μm pore size filters. Syringes and needles were soaked and washed in pyrogen free water for 24 hours. Sterilisation was then achieved by ethylene oxide (Sterivac 5XL, 3M, St Paul, USA) at 55°C for 60 minutes at 100mBar. 5ml universal containers were sterilised by ethylene oxide.

2.2.2 Preparation of radioisotope

Calcium Chloride 45 (37MBq in 0.5ml) was diluted with 3.0ml of normal saline in a class I laminar flow cabinet. The diluted solution was mixed thoroughly. The solution was divided equally into eight aliquots of 4.625MBq. These aliquots were aspirated into eight 1ml syringes.

2.2.3 Administration of radioisotope

The skin of the pregnant female mice was cleaned with alcohol swabs. $^{45}CaCl_2$ was administered subcutaneously under the scruff at day 14 of gestation.

2.2.4 Preparation of particles

All particles were prepared at the Department of Microbiology, University of Leeds.

2.2.4.1 Preparation of polyethylene particles

Aseptic technique was utilised throughout the preparation of the fractionated polyethylene particles. 0.1, 1.0 and 10μm pore filter membranes were labelled individually. Using sterile filter forceps, the filters were transferred to clean sterile 60mm Petri dishes and dried under infra-red lamps for three to four hours. The Petri dishes were sealed and stored in an airtight box containing silica gel until ready for weighing. Using an ion generator (Stat- Attack) to neutralise static, each filter was weighed on a 6-place analytical balance. Each filter was weighed three consecutive times until a reading with an accuracy of 10μg had been obtained. The dishes were then re-sealed and stored in an airtight box containing silica gel. The mean weight for each filter was calculated. Working within a Class II laminar flow cabinet, a very small quantity of Ceridust® polyethylene powder was transferred to a sterile glass Petri dish. A small volume (2-3ml) of sterile, endotoxin-free water containing 0.125% (v/v) 7X detergent was added to the powder whilst mixing thoroughly to disperse the particles. The powder was carefully mixed until the particles were fully wetted and disaggregated. The particles were then transferred to a clean sterile sample pot and diluted with 50 to 100ml of water plus 0.125% (v/v) 7X detergent. The sample pot was sealed and sonicated in a sonicating water bath for 1 hour to further disaggregate clumps of particles. Using sterile filter forceps, the membranes (10, 1.0 and 0.1μm) were

placed in 25mm syringe-tip filter holders. Using clean sterile syringe and sample pots for each particle fraction, the particle suspension was sequentially filtered through the 10μm, 1.0μm and 0.1μm filter membranes. The particle suspension was washed with sterile endotoxin free water. All liquid from the filter holder was removed by blowing air through the membrane. The 0.1μm filter membranes with the particles were retained. The retained membranes were transferred with sterile filter forceps to clean sterile 60mm Petri dishes and dried under infra-red lamps for 3-4 hours. The filter membranes and particles were re-weighed three consecutive times. The average weight of each filter was calculated. The mass of particles on each filter was then calculated.

2.2.4.2 Preparation of alumina ceramic particles

The alumina powder was treated to remove surface endotoxins. The powder was soaked for 24 hours in 1% (w/v) solution of E-Toxaclean. It was then washed 15 times with sterile, pyrogen-free water and heated to 180°C for 5 hours. This has been shown to effectively destroy surface endotoxin (Tsuji *et* al., 1978). The powder was then suspended in hyaluronic acid at $2mg.ml^{-1}$ (v/v).

2.2.5 Preparation of tissue for histology

Tissue processing involves the embedding of tissue in an environment that acts as a support medium. It will then be possible to cut very thin tissue sections, ranging from 4 to 15μm in thickness. Paraffin wax is the most common embedding medium and was the medium of choice for this study.

The processing of tissues involves three steps:

1. Dehydration of tissues. This removes any aqueous fixative and any water present in the tissues.
2. The second stage is called "clearing" of the tissue. This allows effective visualisation of the tissues as the clearing fluid has a similar refractive index as protein, which renders the tissue translucent. Xylene was used in this procedure.
3. The last stage is the embedding of the tissue in molten medium.

2.2.5.1 Dehydration and clearing

The sample was removed from its fixative (10% formalin) and placed in a processing cassette and labelled using pencil, which is not removed by any of the processing fluids. Samples were then placed into a series of graded alcohols, making sure the alcohol can access all parts of the tissue in the cassette. Firstly, the tissue was placed into 70% (v/v) ethanol for a period of 3 to 8 hours, after which it was removed and placed into 90% (v/v) ethanol overnight (16 hours). The tissues are then placed in absolute alcohol for a period of 2 hours followed by a fresh immersion in absolute alcohol for a further 3 hours. A final immersion in fresh alcohol for 3 hours completes the dehydration process. Following dehydration, the tissues are immersed overnight in the clearing agent, xylene. After the clearing process, the tissues are ready for embedding in molten paraffin. There are three stages to this procedure and fresh molten paraffin must be used at each stage. The first stages are immersion in molten paraffin for 1.5 hours, followed by a final immersion for 2 hours. The tissues are then embedded in molten paraffin and allowed to cool.

2.2.5.2 Embedding

The embedding procedure simply sets the tissue in solid paraffin to allow sectioning. The wax mould was filled with molten paraffin up to a level that will cover the tissue section. The wax was then allowed to cool sufficiently. Using forceps, the tissue was placed into the semi-molten wax, whilst orientating the tissue so that the cutting surface is face down. The tissue was held in place until it is held by the solidifying wax and the plastic cassette base was placed on top of the mould and completely filled with molten wax. The mould was then carefully cooled. After the cooling process, the plastic mould is removed.

2.2.5.3 Sectioning

When sectioning the tissues, the block is trimmed on all 4 edges using a scalpel making sure that opposite sides are parallel. The knife is then clamped to the microtome and its tilt set. The amount of tilt used depends on the tissue. The tilt angle is usually set between 3 and 10°. The tissue block is then clamped to the microtome and adjusted so that the leading edge of the block is parallel to the knife edge.

2.2.6 Measurement of bone markers by ELISA

Urine supernatant samples were frozen at -20°C. When required for ELISA, the supernatants were thawed and measured for the presence of NTx and DPD. Both markers were assayed using a commercially available kit.

2.2.7 Quantification of NTx and DPD in urine supernatants

The NTx and DPD ELISA were performed according to the instructions supplied with the assay kits (Quidel Diagnostics). All the necessary reagents were supplied with the kit.

2.2.7.1 Solutions required

Assay calibrators consisting of purified NTx antigen in buffered diluent with the following concentrations – 1, 30, 100, 300, 1000, 3000 nM BCE (Bone Collagen Equivalent).

Standards consisting of DPD purified from bovine bone in 10 mmol/L phosphoric acid with the following concentrations – 0, 3, 10, 30, 100, 300 nmol/L DPD.

2.2.8 Detection and quantification of endotoxin in alumina wear debris using Limulus Amoebocyte Lysate (LAL) test

Pyrotell-T® was freshly reconstituted with endotoxin free water (5ml per vial), gently mixed, covered with Parafilm M® and immediately chilled on ice. Control Standard Endotoxin (*Eschericia coli* 0113:E10 lipopolysaccharide; LPS) was reconstituted with endotoxin-free water to give a stock concentration of 1000 EU/ml, vortexed for 1 minute and then for further periods of 1 minute at 10 minute intervals for a period of 60 minutes. A standard curve was then constructed by diluting the Control Standard Endotoxin to give final concentrations of 1, 0.5, 0.25, 0.125, 0.06 and 0.03 EU/ml. Test samples were prepared neat and diluted (1/2, 1/10, 1/20, 1/30, and 1/40) in endotoxin-free water. To ensure that surface endotoxin was released from the test particles, the debris

was incubated in LAL reagent water for 1 hour at 37°C, then sonicated in a sonicating water bath for a minimum of 1 hour. Positive Product Controls were prepared by "spiking" the neat and diluted samples with endotoxin (at a concentration of 0.125 EU/ml) to detect any enhancement or inhibition caused by the sample. Endotoxin standard dilutions, test samples and Positive Product Controls were then added to 5ml glass test tubes (200µl/tube). Negative controls (endotoxin-free water) were included with each test. All tests were performed in duplicate. Pyrotell-T® (50µl/well) was then added to each tube and the plates gently shaken for 30 seconds prior to incubation at 37°C. The optical density was then read at intervals at a wavelength of 405nm. Results of the standard series were then analysed by linear regression and the time point at which the correlation coefficient was 0.980 or greater was accepted. The concentrations of endotoxin present in the test samples and the Positive Product Controls were then calculated. The dilution at which no enhancement or inhibition were observed was then used to calculate the endotoxin concentration present in the neat sample.

2.2.9 Statistical Analysis

Statistical advice and support was provided by the Department of Epidemiology, University of Leicester.

The following statistical analyses were used.

Mean $\mathbf{X = \frac{\Sigma x}{n}}$

Σ = sum

x = individual value

n = number of replicates

Standard deviation $\mathbf{SD = \sqrt{\frac{(x - X)^2}{n - 1}}}$

Standard error of the mean $\mathbf{SE = \frac{SD}{\sqrt{n}}}$

95% Confidence limits (CL) **95%CL = SE x t (α, v) value**

t = t – value obtained from the Students t-distribution table

α = Level of probability

v = Degrees of freedom (n-1)

Linear Mixed Effects model

A linear mixed effects model was used to examine the difference in outcome (scintillation count per minute per milligram in weight of femur, in units, CPM/mg) between the femora that underwent implantation of ceramic particles and those on which sham surgery (injection of hyaluronic acid only) was performed. A coefficient was provided in the model for each of the litters allowing for inherent random variation between the litters. The outcome of the non-operated femora (control) was also assessed and this was included as a covariate in the analysis. Adjustment was also made for the sex of the mouse, whether surgery was performed on the right or left femur, whether there was leaking of ceramic particles during implantation or injection of hyaluronic acid and whether the femur was fractured. Statistical significance was set at 5% level. The difference in the outcome between the implantation of ceramic particles and sham surgery that was decided to be of clinical significance was set at 50 Counts Per Minute Per Milligram (CPM/mg).

Analysis of NTX and DPD results

Results were plotted on histograms and tested for skewness and kurtosis. Results were skewed and non-parametric tests were applied for analysis. Kruskal-Wallis test was used for analysis of three independent groups. Mann Whitney-U test was used for two independent group analyses. Pearson's correlation coefficient was used for correlation analyses.

CHAPTER 3

QUANTITATIVE ANALYSIS OF WEAR DEBRIS INDUCED OSTEOLYSIS IN OUR ANIMAL MODEL

3.1 INTRODUCTION

The biological response to ultra high molecular weight polyethylene (UHMWPE) wear debris, either alone or in combination with other factors is believed to play an important role in aseptic loosening of metal on UHMWPE total hip replacements (Harris *et al.*, 1976; Mirra *et al.*, 1982; Jasty *et al.*, 1986). UHMWPE wear debris generated at the articulating surfaces enters the peri-prosthetic tissue where it is phagocytosed by macrophages. The macrophages then release pro-inflammatory cytokines and other mediators of inflammation that stimulate osteoclastic bone resorption leading to osteolysis and eventual loosening of the prosthesis (Murray *et al.*, 1990; Santavirta *et al.*, 1990; Jiranek *et al.*, 1993). UHMWPE wear debris has been shown to stimulate osteolytic cytokine release by macrophages *in vitro* and factors such as size and volume of the debris have been shown to be critical in the response (Green *et al.*, 1998; Voronov *et al.*, 1998). The problems associated with metal on polyethylene hip replacements have led to an increased interest in the development and use of alternative bearing surfaces such as ceramic on ceramic hip prostheses. Lerouge *et al* (1996) compared the histology of tissues from around ceramic on ceramic prostheses with tissues from metal on UHMWPE total hip replacements and concluded that there was no significant difference between the two groups in relation to the cellular response (presence of macrophages, neutrophils and giant cells).

Various *in vivo* models of osteolysis have been described. These include the application of titanium wear particles (1-3μm in diameter) to murine calvaria then measuring the effect of sagittal suture width and osteoclasts (Schwarz et al, 2000).

These outcomes are either surrogates or indirect endpoints of the measurement of osteolysis. Another model described by Allen et al (1996) uses an implant and additional particles in a rat knee and measures the volume of periprosthetic bone histologically and the thickness of the fibrous tissue response. This model is semi-quantitative. The making of implants to fit a small animal is unduly complex and expensive. Our model uses particles only, which is simple and inexpensive. Radiolabelled calcium chloride ($^{45}CaCl_2$) accurately quantifies osteolysis using simple scintillation counting.

The aim of this part of the study was to develop an *in vivo* model of wear debris osteolysis in a small animal and to determine whether sham surgery influenced the uptake of $^{45}CaCl_2$.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

3.2.1.1 Animals

All animals were bred at the Division of Biomedical Services at the University of Leicester. Two sets of animals were utilised in this experiment.

The first set of animals consisted of seven female mice that were time mated and produced a total of 52 offspring. The offspring details are given in Table (3.1).

The second set of animals consisted of six female mice that were time mated and produced a total of 47 offspring. The offspring details are given in Table (3.2).

After the delivery of the offspring, the female time mated mice were not utilised in any further experiment.

Both female and male offspring were kept with their mother until weaning (up to 3 weeks). Following weaning, all female offspring were placed in a single cage and all male offspring placed in a separate cage. During the separation of the sexes from each mother, the animals were coded by ear marks. The animals were then left alone without any intervention until the age of 10 weeks.

Animals were either implanted with ceramic particles that was suspended in hyaluronic acid or injected with hyaluronic acid only (sham surgery).

Table 3.1

First set of animals

Litter Number	Number of Offspring	Male Offspring	Female Offspring
1	3	2	1
2	4	3	1
3	7	2	5
4	11	6	5
5	10	7	3
6	8	3	5
7	9	5	4
Total	**52**	**28**	**24**

Table 3.2

Second set of animals

Litter Number	Number of Offspring	Male Offspring	Female Offspring
1	6	2	4
2	8	5	3
3	8	3	5
4	8	4	4
5	7	2	5
6	10	2	8
Total	**47**	**18**	**29**

3.2.1.2 Anaesthetic Equipment

All surgical procedures took place in the surgical theatre at the Division of Biomedical Services at the University of Leicester.

The anaesthetic apparatus consisted of an induction box, oxygen cylinder, isoflurane container, scavenger, tubing and facemask (Figures 3.1 and 3.2). All procedures were undertaken on a plastic tray (Figure 3.3). The facemask was secured to the plastic tray. All drapes were supplied by the Division of Biomedical Services.

3.2.1.3 Surgical Equipment

3.2.1.3.1 Disposable scalpels

Sterile disposable scalpels were supplied by Swann-Morton Ltd. (Sheffield, UK). The scalpels were fitted with number 15 blades.

3.2.1.3.2 Needles and syringes

100 microlitre syringes and needles were supplied by VWR International (Dorset, UK). One millilitre syringes were supplied by Plastipak (Madrid, Spain). 25 gauge needles (0.5 x 25mm) were supplied by Terumo (Leuven, Belgium).

3.2.1.3.3 Suture material

5/0 blue polyamide monofilament non-absorbable suture (Ethilon®) was supplied by Ethicon (Livingston, UK).

3.2.1.3.4 Surgical sets

All surgical sets which included forceps, scissors, needle holders and disposable swabs were provided by the Division of Biomedical Services, University of Leicester.

3.2.1.4 Alumina and polyethylene particles

Both ceramic alumina and polyethylene particles were supplied by the Department of Microbiology, University of Leeds.

3.2.1.5 Hyaluronic acid and iodine

Sodium hyaluronate 20mg/2.0ml (Fermathron™) in pre-filled syringes were donated by Biomet, Merck (South Wales, UK). Povidone-Iodine USP 7.5% w/v (Betadine®) was supplied by Seton Healthcare Group plc (Oldham, UK).

3.2.1.6 Fluids and analgesia

Normal saline (0.9%) was supplied by Baxter (Northamptonshire, UK). Meloxicam (Metcam™) and buprenorphine (Temgesic™) were both supplied by Pfizer Ltd. (Surrey, UK).

3.2.1.7 Scintillation fluid and vials

Optiphase Hisafe 3 scintillation fluid was purchased from Fisher Scientific (Leicestershire, UK). Twenty millilitre scintillation vials were donated by Isotech Services Ltd. (Leeds, UK).

3.2.1.8 Scintillation counter and weighing scale

The scintillation counter (Packard 1500, TRI-CARB liquid Scintillation Analyzer) used for counting was manufactured by Packard, IL, USA. A five decimal balance (Sartorius 1700) was used for weighing the femora bone was manufactured by Sartorius Werke (Göttingen, Germany).

Figure 3.1

Anaesthetic Induction Box

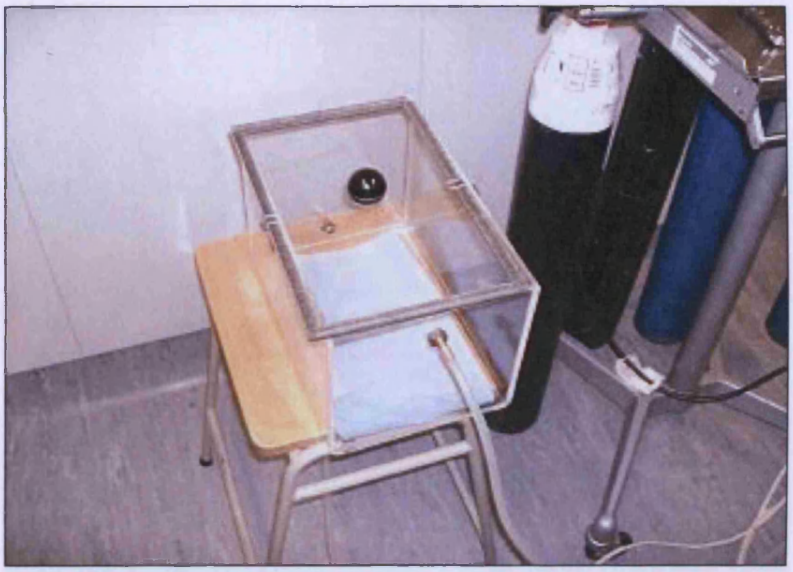


Figure 3.2

Anaesthetic Apparatus

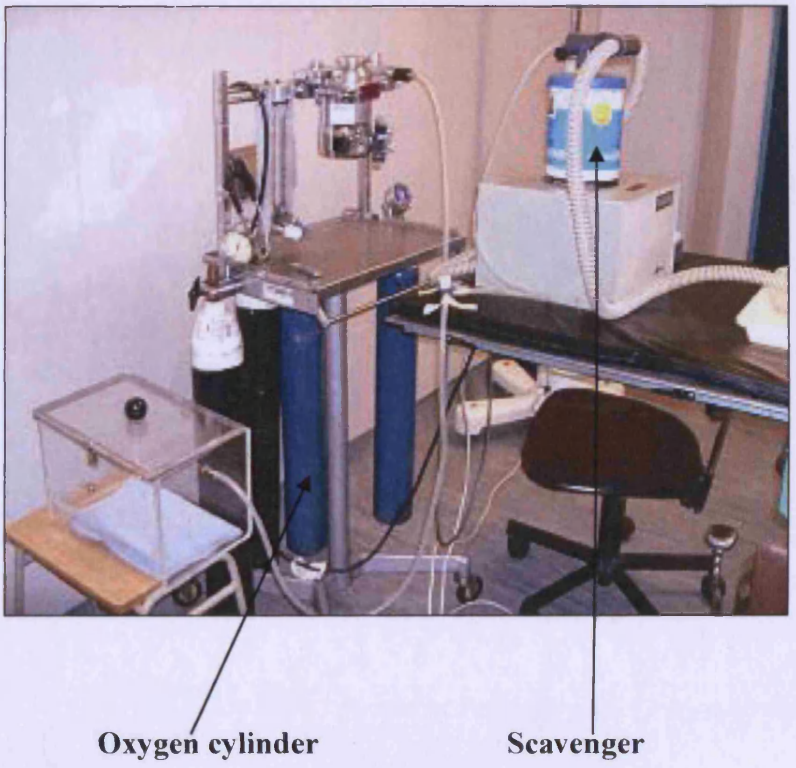
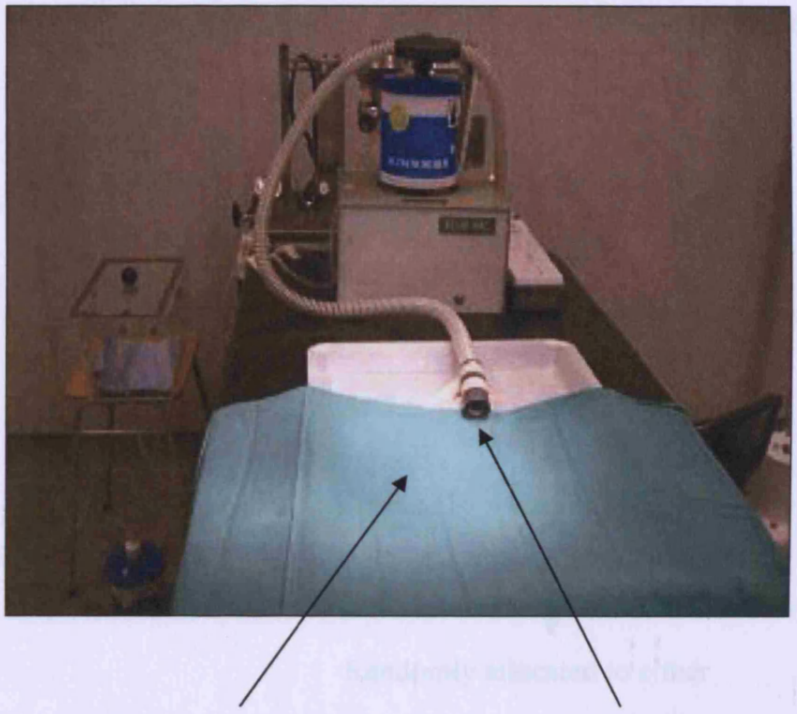


Figure 3.3

Operating Table



3.2.2 METHODS

3.2.2.1 Flow diagrams

The following flow diagrams display the outline used in this study.

Figure 3.4

Outline of the study for the first set of animals

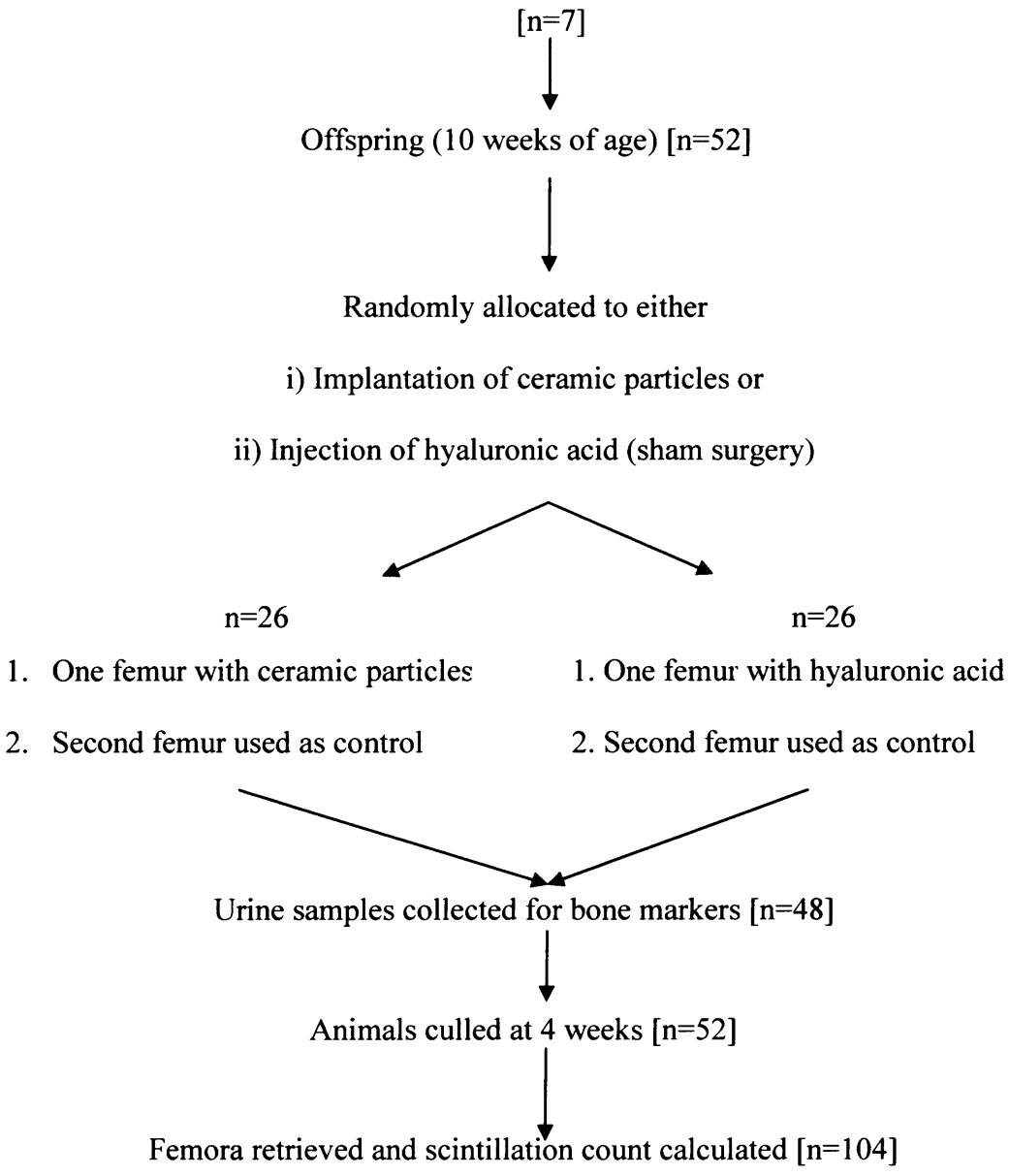
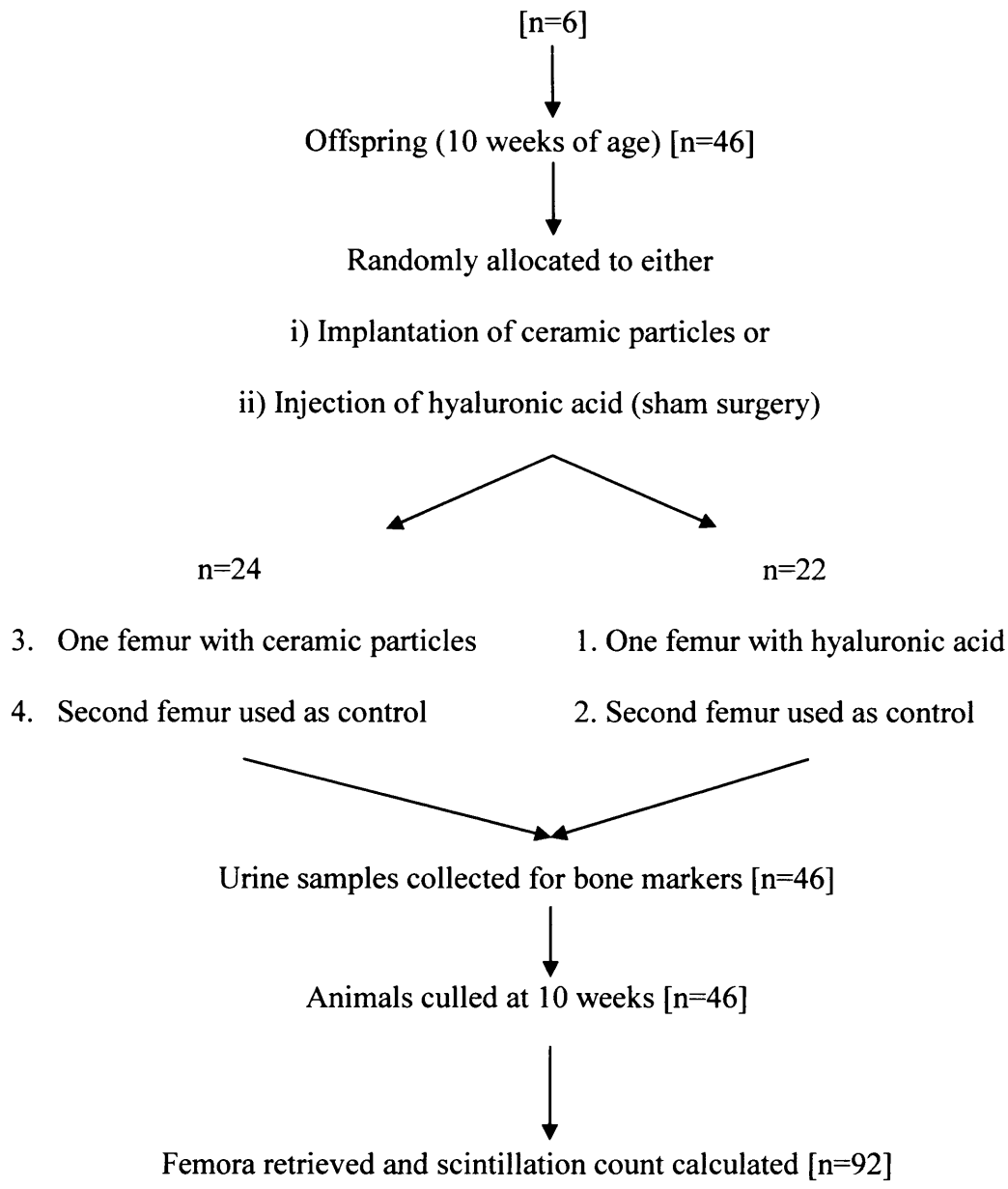


Figure 3.5

Outline of the study for the second set of animals

Time mated female mouse injected with radioactive calcium (day 14 of gestation)



3.2.2.2 Administration of radioisotope

All pregnant female mice were injected with radioisotope calcium 45 ($^{45}CaCl_2$) subcutaneously on day 14 of gestation. The females were checked the following day after mating for vaginal plugs to confirm successful fertilisation. The offspring were not injected with any radioisotope.

The uptake of radioactive calcium in the pregnant mice was determined by culling the first eight female mice after weaning their offspring. Both humeri and femora of the mice were collected using different scalpels for each bone. The bone was inserted into a container that was labelled and contained 3ml of 1M hydrochloric acid (HCl). Bone were left in HCl overnight at room temperature until all bone dissolved. The preparation of scintillation fluid and counting is described in sections 3.2.2.8 and 3.2.2.9 respectively.

3.2.2.3 Preparation of alumina and polyethylene particles

The techniques utilised to prepare both alumina and polyethylene particles are described in section 2.2.4. Alumina particles were suspended in hyaluronic acid (2mg/ml) giving a final concentration of ceramic particles of 2.5mg/ml.

3.2.2.3.1 Characterisation of alumina powder

The alumina debris was first characterised by filtering a small fraction through a 0.1μm pore size polycarbonate filter, followed by examination by SEM (Hitachi S3000). Ten micrograms of the alumina powder was added to 1ml pyrogen free water in a clean glass universal container and sonicated for 1 hour in a sonicating water bath to separate particles. Two hundred microlitres of powder solution was filtered onto a 0.1μm membrane filter and placed under an infra-red lamp until

dry. A small section of the filter containing the powder was removed and examined by SEM. The images were analysed using image analysis software (Image Pro Plus, Media Cybernetics, USA) and lengths of 512 particles were recorded.

3.2.2.4 Endotoxin testing

The technique used to analyse endotoxin level in alumina particles is described in section 2.2.8.

3.2.2.5 Administration of ceramic particles or hyaluronic acid (sham surgery)

The two sets of animals were randomly allocated to either the implantation of ceramic particles or injection of hyaluronic acid only (sham surgery). The randomisation was performed using the Excel package (Microsoft Office, Microsoft, USA). Both the litters and mice within each litter were sorted into a random order. Then the four groups (by combination of left/right and implantation of particles/sham surgery) were allocated randomly. Randomisation was 'blocked' within each litter.

3.2.2.5.1 Anaesthetic induction of animal

The mouse was placed in the induction box (Figure 3.1) that delivers a high flow of oxygen and isoflurane [3-4%] (inhalation anaesthesia). The mouse was kept in the induction box until it became drowsy and was unable to move around inside the induction box. Following this, the mouse was removed from the induction box and weighed. The operative site was clipped and the mouse was returned to the

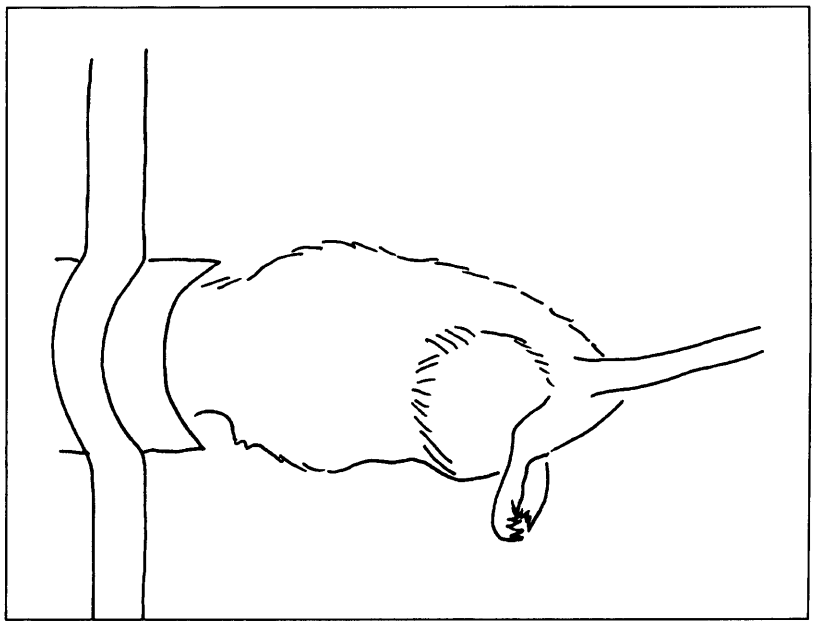
induction box. The mouse was then delivered a high flow of oxygen and isoflurane until it became drowsy again.

3.2.2.5.2 Transfer to operating table

The mouse was then transferred to the operating table (Figure 3.3). The mouse was placed on its side with the operating side facing the ceiling. The mouse's upper airway was fitted to the facemask that delivered oxygen (1-1.5L/min) and isoflurane [2 – 3%] (Figure 3.4). The complete procedure was undertaken using inhalation anaesthesia. The skin of the mouse was cleaned with betadine® and draped. Before the initiation of surgery, the mouse was given 1ml of subcutaneous Normal Saline and Temgesic™ (0.1mg/kg) at an appropriate dose according to weight. Deep pinch reflex was tested to make sure that the mouse was in deep anaesthesia before surgery.

Figure 3.6

Mouse attached to facemask

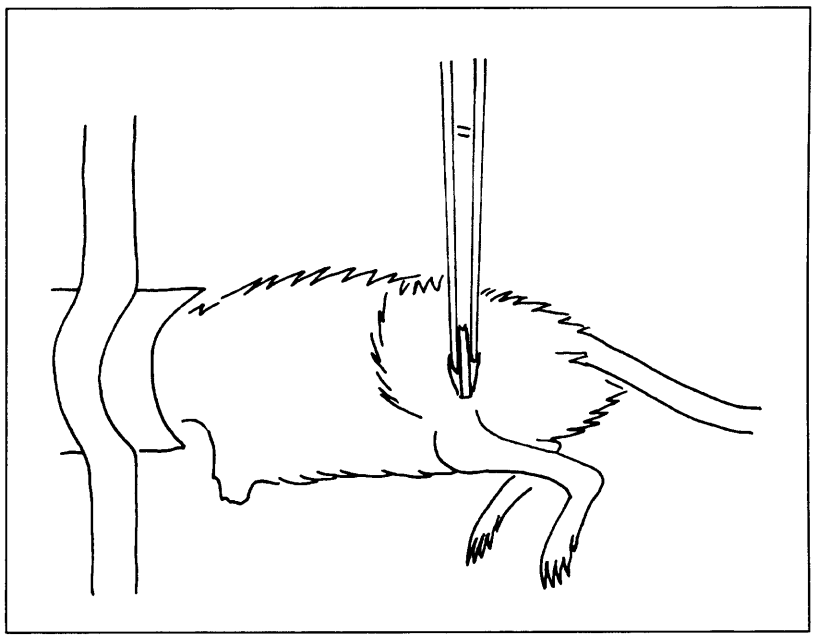


3.2.2.5.3 Exposure and dissection of femur

An incision was made over the proximal aspect of the femur and along it's length using a scalpel. Sharp dissection was used to expose the abductors of the hip and tip of the greater trochanter. The proximal aspect of the femur was also exposed and held with forceps (figure 3.5) to identify the direction of the femur. Bleeding was very minimal and diathermy was not required during the procedure.

Figure 3.7

Dissection of femur



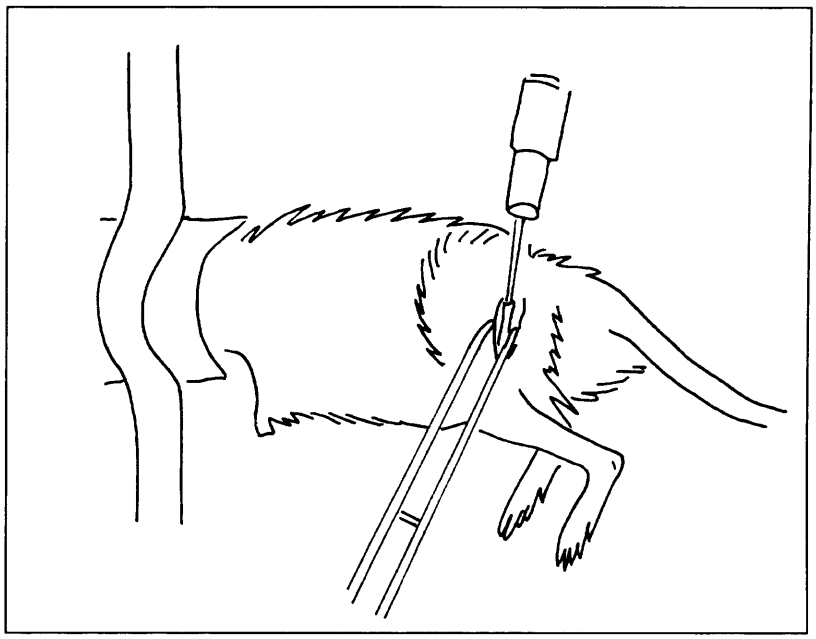
3.2.2.5.4 Entry point to the femur

Following exposure of the proximal femur and holding the proximal shaft with forceps to identify the direction of the femur, a 25 gauge needle was used to create an entry point in the piriformis fossa of the femur (Figure 3.6). The needle was then advanced down the femoral shaft under direct vision. The needle could be

visualised through the cortex of the femur. The needle was small enough to be advanced down the femur but not large enough to pierce the cortex. Following the advancement of the needle in the proximal femur, the needle was twisted to confirm that the leg was moving with the needle as one unit.

Figure 3.8

Entry point to femur



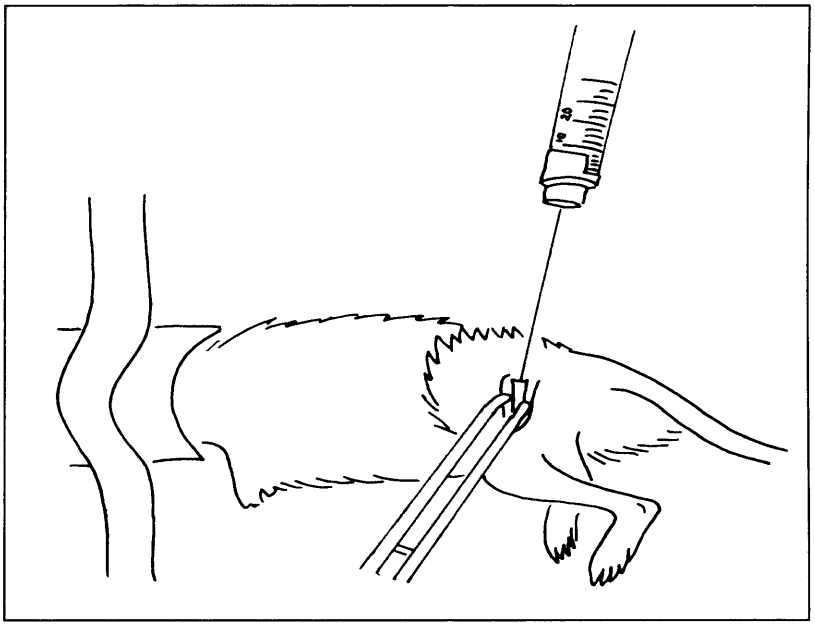
3.2.2.5.5 Implantation of ceramic particle or injection of hyaluronic acid

After making the entry point in the proximal femur, the microlitre needle (gauge 26s) was introduced into the medullary canal of the femur. The syringe was attached to a 100μL syringe that contained either ceramic particles suspended in hyaluronic acid or hyaluronic acid alone. The ceramic particles or hyaluronic acid was aspirated into the syringes after priming. The solutions came from one master solution and a separate needle was used on each occasion. The volume in the

needle was specified by the manufacturer. Once the needle was in the medullary canal, 20μL of either ceramic particles or hyaluronic acid was injected. A separate needle was used for each mouse and needles were used solely for the injection of one material only. The syringes were also used solely for one material and not interchanged during this experiment. The injection was performed under direct vision.

Figure 3.9

Implantation of ceramic particles/hyaluronic acid into femur



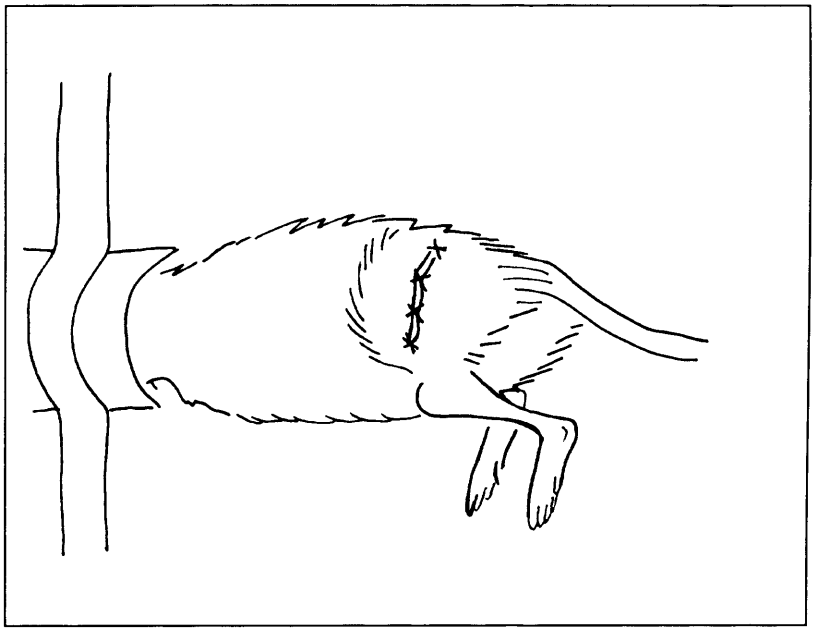
3.2.2.5.6 Closure of wound

Following the implantation of ceramic particles or injection of hyaluronic acid, the syringe was removed from the medullary canal. Any leak that occurred during this procedure was seen and documented. The skin was closed with 5/0 prolene

using simple interrupted sutures. Any wounds that dehisced due to disruption of sutures by the mouse were re-sutured the same day under general anaesthetic.

Figure 3.10

Closure of wound



3.2.2.6 Post operative care

Following the surgical procedure, betadine® was cleaned off the mouse with a swab soaked in warm water. Mice were placed into individual cages with infra-red lights shimming onto their cages and cages were placed on heated mats. Mice had daily post-operative checks and were given metcam™ for the first three days post-operative. Mice were then transferred to their ordinary cages after three days following their surgery.

3.2.2.7 Femora bone retrieval

Mice were culled either after 4 or 10 weeks according to their respective set. All mice were culled using schedule 1 procedures. Following culling, incisions were made over the femora and bone was dissected out of the carcases. Two different scalpel blades were used for each femora. All soft tissue was stripped off the femora. Femora were cut proximally at the level of the femoral neck and the supra-condylar region distally.

Femora were then placed in containers that were labelled and then transferred to the laboratory for weighing. Each femora was weighed three times using Sartorius 1700 scale and an average was then calculated.

3.2.2.8 Dissolution of femora bone

Each femora was inserted into a container that was labelled and contained 3ml of 1M hydrochloric acid (HCl). Femora were left in HCl overnight at room temperature until all bone dissolved.

3.2.2.9 Preparation of scintillation fluid

One millilitre sample of the dissolved femora taken from the container with a pipette was transferred to a scintillation vial containing 15 ml of scintillation fluid. The cap of the vial was labelled and the vial was then gently shaken to ensure the sample and scintillation fluid were mixed.

3.2.2.10 Scintillation counting of bone

The scintillation vial was placed into the scintillation counter and read three times. Each reading was five minutes in duration. The average of the three counts per minute (CPM) was taken as the final reading. The final reading was then divided by the weight of the femora in milligrams to produce a final outcome of counts per minute per milligram (CPM/mg). The samples from each set of animals were analysed on the same day to ensure that the half-life of the radioisotope did not influence the results.

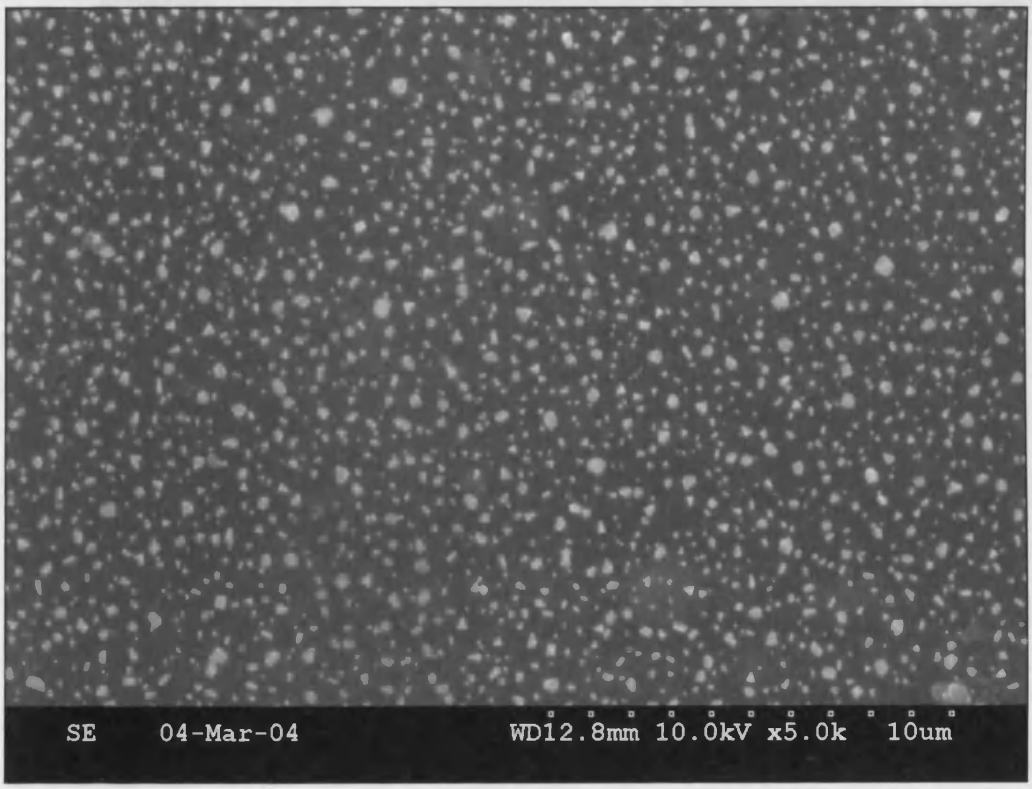
3.3 RESULTS

3.3.1 SEM of alumina wear debris

The mean size of the particles was 0.298μm, range: 0.028 – 0.59μm. The SEM appearances of the alumina powder is shown in Figure 3.9 and the particle size distribution is shown in Figure 3.10. All the particles had a uniform, polygonal morphology. We were able to process a large quantity of alumina powder that could be used for the implantation of the murine femora.

Figure 3.11

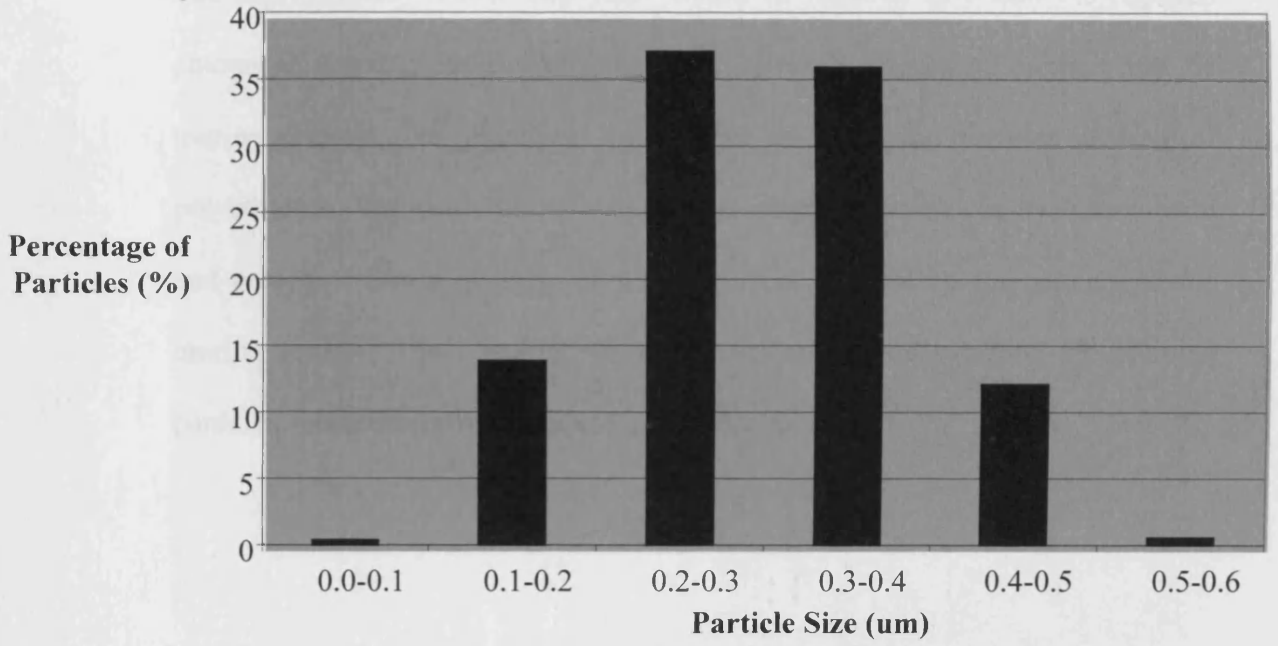
SEM image of alumina powder



The particles were uniform in size and shape.

Figure 3.12

Frequency distribution of alumina particles as a function of size



The majority of the particles were 0.2 – 0.4μm in size.

3.3.2 SEM of polyethylene wear debris

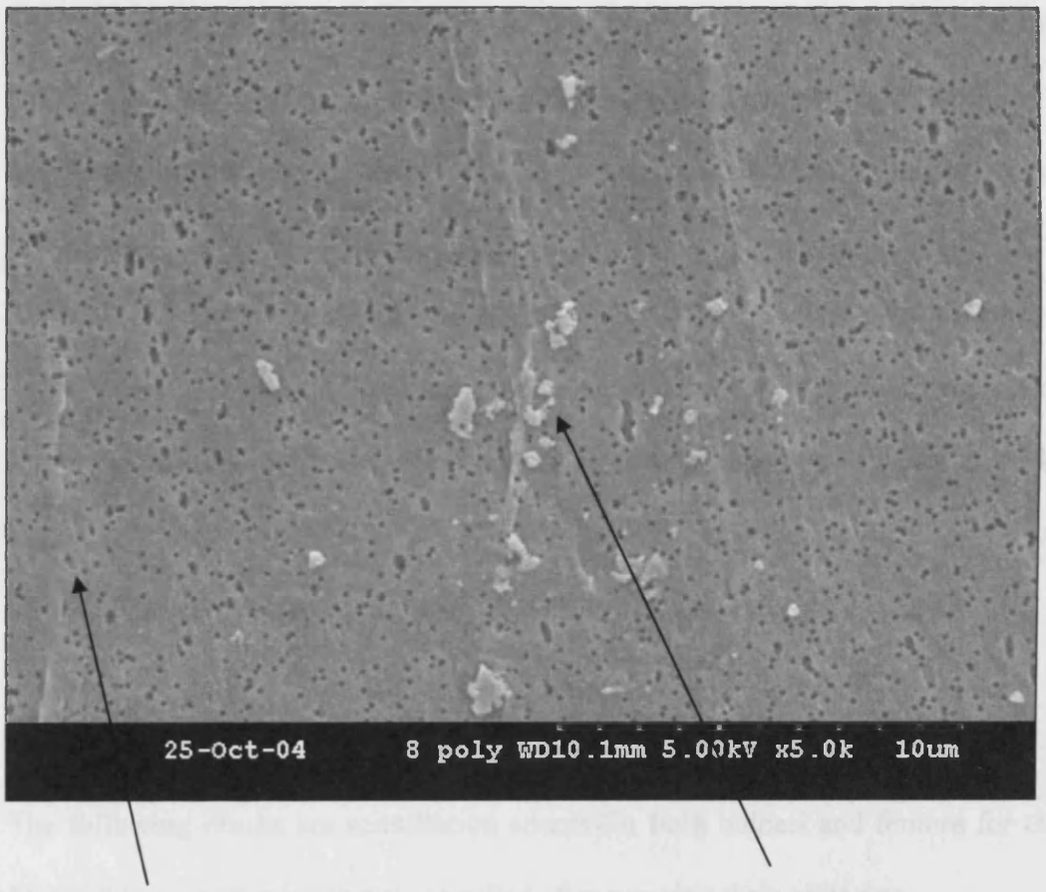
The particle size was clinically significant as shown in Figure 3.11.

Unfortunately we were not able to obtain enough quantity of polyethylene debris to implant into the femora of the animals. Several months were spent trying to obtain enough quantity of polyethylene debris. Some of the difficulties encountered with polyethylene debris was the difficulty to suspend the particles in Normal Saline or hyaluronic acid. We also tried to suspend the polyethylene debris in other solutions such as 50% dextrose and dextrose/saline. The particles adhered to the surface of these solutions due to their low density and it was not possible to suspend the particles in solution. The particles also adhered to glass

and plastic materials used. The wetting and disaggregation of the polyethylene powder was a time consuming and technically difficult process. It was predicted that approximately one to two years would be required to obtain the required amount of polyethylene particles that were clinically relevant to implant into the murine femora. We therefore decided to use alumina particles instead of polyethylene. We were able to suspend the alumina particles in hyaluronic acid and process a larger quantity of wear debris that could be implanted into the murine femora. The handling, disaggregation and processing of the alumina particles was technically easier and more efficient.

Figure 3.13

SEM image of polyethylene powder



0.1μm sized pore of filter

Polyethylene particles

3.3.3 Endotoxin tests

Alumina powder debris was tested for endotoxin levels and was shown to be less than 0.01 endotoxin units (EU) per ml (0.001732 EU/ml). The lowest standard in the test was 0.000977 EU/ml. These levels represented values inside the detection limit of the test which was approximately 0.001 EU/ml.

3.3.4 Complications of surgery

3.3.4.1 Perioperative deaths

Only one mouse died following surgery. The mouse was from the second set of animals and belonged to litter 6. The mouse underwent injection of hyaluronic acid into the right femur. There were no perioperative complications during the procedure.

3.3.4.2 Wound infections and fractures

There were no mice that developed either superficial or deep wound infections following surgery. All fractures were discovered at necropsy of the animals. Leaking of ceramic particles or hyaluronic acid during implantation or injection into the femora is recorded in the respective results sections.

3.3.5 Scintillation counts of pregnant murine bone

The following results are scintillation counts for both humeri and femora for the first eight pregnant mice that were culled after weaning their offspring.

Table 3.3

Bone	Mean scintillation count	Standard deviation of scintillation count
Right femur (n=8)	3807	1302
Left femur (n=8)	3959	1385
Right humerus (n=8)	4714	1709
Left humerus (n=8)	4911	1827

The following results could be used as a guide to perform sample size calculations for future studies investigating uptake of radioactive calcium in murine bone. These pregnant mice were culled so that we could determine the scintillation counts in different bones in the mice that were injected with radioactive calcium.

3.3.6 Scintillation counts of femora

3.3.6.1 First set of animals

The first set of animals were culled 4 weeks after implantation of ceramic particles or injection of hyaluronic acid (sham surgery)

3.3.6.1.1 Scintillation counts of animals with ceramic particles

The following results are the counts per minute per milligram of bone (CPM/mg) of the animals that had ceramic particles implanted into their femora.

Mouse number 23 sustained a fracture of the femur during the implantation of ceramic particles

Mice numbers 7, 13, 22, 48 and 50 had a leak of ceramic particles into the adjacent soft tissue during implantation of ceramic particles.

Table 3.4

Litter Number	Mouse Number	CPM/mg (Operated femur)	CPM/mg (Non operated femur)
1	2	526.73	625.46
2	4	542.23	588.74
2	5	553.26	582.37

3	6	495.42	530.77
3	7	470.07	515.53
3	8	382.14	461.55
4	9	355.35	394.06
4	10	263.35	282.90
4	11	330.08	363.85
4	12	335.05	457.38
4	13	236.65	350.49
4	14	278.08	312.99
5	15	297.46	325.57
5	16	347.73	363.55
5	17	314.86	344.73
5	18	317.49	345.38
5	19	309.59	367.59
6	20	361.91	486.55
6	21	320.69	450.94
6	22	240.64	309.35
6	23	215.97	424.68
7	24	301.19	415.98
7	25	379.21	426.49
7	48	253.55	369.94
7	49	345.98	407.28
7	50	356.12	426.43

Mean CPM/mg of operated femora: 351.15

95% Confidence Interval of operated femora: 313.99 – 388.31

Standard Deviation of operated femora: 94.33

Mean CPM/mg of non operated femora: 420.41

Standard Deviation of non operated femora: 90.99

3.3.6.1.2 Scintillation counts of animals with sham surgery

The following results are the counts per minute per milligram of bone (CPM/mg) of the animals that had injection of hyaluronic acid (sham surgery) into their femora.

Mice numbers 38 and 40 sustained fractures of their femora during the injection of hyaluronic acid (sham surgery).

Mice numbers 1, 6, 12, 18, 32, 38 and 51 had a leak of hyaluronic acid into the adjacent soft tissue during injection of hyaluronic acid.

Table 3.5

Litter Number	Mouse Number	CPM/mg (Operated femur)	CPM/mg (Non operated femur)
1	1	466.37	573.49
1	3	642.68	698.43
2	5	512.53	596.85
2	6	583.95	664.72

3	7	491.33	575.74
3	8	506.17	538.71
3	9	479.95	559.19
3	10	407.78	445.54
4	11	368.52	428.90
4	12	389.21	428.55
4	13	311.70	390.37
4	14	402.95	412.73
4	15	353.86	354.09
5	16	281.23	345.00
5	17	360.91	354.55
5	18	352.53	389.03
5	31	328.18	375.03
5	32	318.37	317.48
6	36	331.54	417.86
6	37	265.07	335.10
6	38	244.59	434.15
6	39	366.70	453.46
7	40	294.04	412.35
7	47	342.48	363.81
7	51	294.09	379.42
7	52	379.91	435.80

Mean CPM/mg of operated femora: 387.56

95% Confidence Interval of operated femora: 350.43 – 424.76

Standard Deviation of operated femora: 99.38

Mean CPM/mg of non operated femora: 449.25

Standard Deviation of non operated femora: 104.09

3.3.6.2 Second set of animals

The second set of animals were culled 10 weeks after implantation of ceramic particles or the injection of hyaluronic acid (sham surgery)

3.3.6.2.1 Scintillation counts of animals with ceramic particles

The following results are the counts per minute per milligram of bone (CPM/mg) of the animals that had ceramic particles implanted into their femora.

Mouse number 36 sustained a fracture of the femur during the implantation of ceramic particles

Mice numbers 14, 31 and 41 had a leak of ceramic particles into the adjacent soft tissue during implantation of ceramic particles.

Table 3.6

Litter Number	Mouse Number	CPM/mg Operated Femur	CPM/mg Non operated Femur
1	1	445.94	459.63
1	3	373.24	515.85

1	4	455.03	540.06
2	5	266.46	337.03
2	6	270.08	328.49
2	7	296.84	337.03
2	8	296.06	369.49
3	9	222.28	267.33
3	10	229.54	288.94
3	11	300.43	362.96
3	12	265.57	299.36
4	13	318.39	451.85
4	14	301.99	366.61
4	15	378.63	465.25
4	16	392.30	467.88
5	31	423.48	522.69
5	32	449.44	604.76
5	33	432.22	613.54
5	36	487.06	620.74
6	37	250.67	347.12
6	38	294.97	348.89
6	39	319.56	373.21
6	40	294.33	367.53
6	41	374.08	407.86

Mean CPM/mg of operated femora: 339.11

95% Confidence Interval of operated femora: 305.96 – 372.26

Standard Deviation of operated femora: 78.49

Mean CPM/mg of non operated femora: 419.34

Standard Deviation of non operated femora: 105.14

3.3.6.2.2 Scintillation counts of animals with sham surgery

The following results are the counts per minute per milligram of bone (CPM/mg) of the animals that had injection of hyaluronic acid (sham surgery) into their femora.

No mice sustained fractures of their femora during the injection of hyaluronic acid (sham surgery).

Mice numbers 12 and 13 had a leak of hyaluronic acid into the adjacent soft tissue during injection of hyaluronic acid.

Table 3.7

Litter Number	Mouse Number	CPM/mg Operated femur	CPM/mg Non operated femur
1	2	418.90	541.97
1	4	475.84	520.33
1	5	474.51	509.83
2	6	337.32	373.58

2	7	285.48	322.83
2	8	252.86	313.49
2	9	224.09	354.13
3	10	230.77	283.75
3	11	269.85	323.71
3	12	199.42	270.16
3	13	240.62	331.83
4	14	309.69	372.89
4	15	313.55	399.57
4	16	401.39	440.15
4	17	324.17	415.69
5	18	488.14	582.51
5	19	471.24	616.52
6	20	294.35	365.92
6	21	302.70	353.78
6	22	471.11	404.39
6	23	364.26	377.37
6	24	336.44	384.29

Mean CPM/mg of operated femora: 340.31

95% Confidence Interval of operated femora: 299.33 - 381.29

Standard Deviation of operated femora: 92.41

Mean CPM/mg of non operated femora: 402.67

Standard Deviation of non operated femora: 95.24

3.3.7 Summary of results of first set of animals

The number of mice implanted with ceramic particles - mean (standard deviation) of CPM/mg by litter and treatment

Table 3.8

Litter Number	Number of mice	Ceramic particles Outcome (CPM/mg)	Control Femur Outcome (CPM/mg)
1	1	526.73	625.46
2	2	547.74 (7.79)	585.55 (4.51)
3	3	449.21 (59.45)	502.61 (36.37)
4	6	299.76 (46.99)	360.28 (61.48)
5	5	317.43 (18.61)	349.36 (16.86)
6	4	284.80 (68.12)	417.88 (76.67)
7	5	327.21 (49.99)	409.22 (23.38)
Total	**26**	**351.18 (94.33)**	**420.41 (90.99)**

The number of mice injected with hyaluronic acid - mean (standard deviation) of CPM/mg by litter and treatment

Table 3.9

Litter Number	Number of mice	Sham surgery Outcome (CPM/mg)	Control Femur Outcome (CPM/mg)
1	2	554.52 (124.66)	635.96 (88.35)
2	2	548.24 (50.50)	630.78 (47.99)
3	4	471.31 (43.69)	529.80 (58.18)
4	5	365.25 (35.37)	402.93 (31.50)
5	5	328.24 (31.48)	356.22 (27.66)
6	4	301.97 (56.92)	410.14 (52.10)
7	4	327.63 (41.66)	397.85 (32.40)
Total	**26**	**387.56 (99.38)**	**449.25 (104.09)**

3.3.8 Summary of results of second set of animals

The number of mice implanted with ceramic particles – mean (standard deviation) of CPM/mg by litter and treatment

Table 3.10

Litter Number	Number of mice	Ceramic particles Outcome (CPM/mg)	Control Femur Outcome (CPM/mg)
1	3	424.74 (44.83)	505.18 (41.27)
2	4	282.36 (16.34)	343.01 (18.10)
3	4	254.46 (36.03)	304.65 (41.10)
4	4	347.83 (44.32)	437.90 (48.04)
5	4	448.05 (28.16)	590.43 (45.63)
6	5	306.72 (45.10)	368.92 (24.56)
Total	**24**	**339.11 (78.49)**	**419.34 (105.14)**

The number of mice injected with hyaluronic acid - mean (standard deviation) of CPM/mg by litter and treatment

Table 3.11

Litter Number	Number of mice	Sham surgery Outcome (CPM/mg)	Control femur Outcome (CPM/mg)
1	3	456.42 (32.50)	524.04 16.39)
2	4	274.94 (48.56)	341.01 (27.81)
3	4	235.17 (29.04)	302.36 (30.04)
4	4	337.20 (43.23)	407.08 (28.24)
5	2	479.69 (11.95)	599.51(24.05)
6	5	353.77 (71.27)	377.15 (19.14)
Total	**22**	**340.31 (92.41)**	**402.67(95.24)**

3.3.9 Statistical analysis at 4 weeks

Results of mixed-effects model for the analysis of outcome following surgery (CPM/mg)

Table 3.12

Variable	Estimate	95% CI	P value
Surgery with particles	29.11	4.66 - 53.55	0.02
Leak	26.39	2.64 – 55.43	0.075
Fracture	74.58	18.28 – 130.87	<0.001

From the analysis it was estimated that the mean effect on outcome of implantation of ceramic particles relative to sham surgery was estimated at 29.11 CPM/mg and the true value is likely to be somewhere in the range of 4.66 to 53.55 CPM/mg and this was statistically significant but not clinically relevant (Outcome difference of clinical relevance was set at 50 CPM/mg).

The other covariate significant at the 5% level in this model was whether the femur that underwent surgery had been fractured which tended to decrease the scintillation count by 74.58 CPM/mg. This could be explained by the fact that callus was formed and this increased the weight of the bone and overall reduced the final scintillation count. Leak of ceramic particles or hyaluronic acid had no statistical significance on the scintillation count.

There was evidence that sham surgery affected the scintillation count of the femur relative to the non operated femur of the same animal. The mean size of this effect was estimated to be by 61.01 CPM/mg (95%CI: 36.77 to 85.24; $p < 0.001$).

3.3.10 Statistical analysis at 10 weeks

Results of mixed-effects model for the analysis of outcome following surgery (CPM/mg)

Table 3.13

Variable	Estimate	95% CI	P value
Surgery with particles	11.85	-14.04 – 37.75	0.37
Leak	18.03	-24.80 – 60.87	0.41
Fracture	43.44	-51.89 – 138.74	0.37

From the analysis it was estimated that the mean effect on outcome of implantation of ceramic particles relative to sham surgery was estimated at 11.85 CPM/mg and the true value is likely to be somewhere in the range of -14.04 to 37.75 CPM/mg and this was not statistically significant.

The other covariates (leak and fracture) were not significant at the 5% level in this model.

There was evidence that sham surgery affected the scintillation count of the femur relative to the non operated femur of the same animal. The mean size of this effect was estimated to be by 67.27 CPM/mg (95%CI: 42.97 to 90.94; $p < 0.001$).

3.4 DISCUSSION

The aim of this part of the study was to develop a simple *in vivo* model of wear debris induced osteolysis that will allow the testing of potential pharmaceutical agents against aseptic loosening. Our model intended to use particles only, which is simple and inexpensive but used radiolabelled calcium to accurately quantify osteolysis using simple scintillation counts. Our initial plan was to use polyethylene particles, however, we were not able to process a large enough quantity of these particles to be used in our study. Instead, we opted to use alumina particles. We were able to process a larger amount of ceramic particles and suspend the particles in hyaluronic acid. The particles had to be in a solution form so that they could be implanted into the femur. Suspending polyethylene particles into solution proved to be difficult. The polyethylene powder also adhered to most of the instruments and equipment.

We are not aware of any studies that have used particles in radiolabelled femora to quantify osteolysis. Our model used the mouse instead of a larger animal. Radioactive calcium 45 has a half life of 163 days and this was a limiting factor. The scintillation counts in the second set of animals was less than the first due to the half life of radioactive calcium. We were interested in the difference in scintillation counts between the femora that underwent implantation of ceramic particles and those that underwent injection of hyaluronic acid. The final difference between the groups was more important rather than the absolute scintillation count. The scintillation count of the non-operated femora was incorporated into the model to adjust for the different uptake of radioactive calcium in different animals. Rats exhibit prolonged growth and bones do not

become fully ossified until their second year, whereas, murine bones ossify much quicker. We waited 10 weeks before operating on the mice so that the animal was of an adequate size to have ceramic particles implanted and that the femur was fully ossified. However; during this 10 weeks of growth, bone remodelling was occurring and this would potentially interfere with the calcium metabolism within the murine femora and decrease the overall uptake of radioactive calcium. Our aim was to compare the scintillation counts between the femora that underwent implantation of ceramic particles and sham surgery, therefore the overall influence of the calcium metabolism during this growth and uptake of radioactive calcium would be constant for both groups of animals but this is a possible shortcoming of the model. The offspring themselves were not injected with calcium 45 because of the results of a previous study in our department (Ong and Taylor, 2004). They found that the uptake of calcium 45 was variable between animals when individually injected. Therefore, to eliminate this variable we decided to inject the mother with calcium 45 so that the uptake of radioactive calcium in the offspring was similar. We found that the uptake of calcium 45 was similar amongst animals within the same litter.

Reynolds and Dingle described an *in vitro* method to investigate bone resorption using radiolabelled calcium 45 murine calvaria. They found this method to be extremely sensitive in the study of hormone induced bone resorption (Reynolds and Dingle, 1970). The radiolabelled murine calvaria model had been adopted and modified by various investigators of aseptic loosening. Horrowitz and Purdon (1995) used radiolabelled calvaria to investigate the effect of wear particle

activated macrophages on bone resorption. They also used a similar model to investigate the pharmacological effect of bisphosphonate on aseptic loosening (Horrowitz *et al*, 1996). Yokohoma *et al* (1995) used dead radiolabelled calvaria to investigate the direct bone resorption activity of wear particle activated macrophages. This method has been widely used for the investigation of bone resorption because it is relatively easy to prepare. In our study, we opted to use the femur instead of the calvaria. One of the most important reasons for this was that we wanted to implant particles into a long bone instead of applying particles onto a flat bone as this simulates joint replacement better. Another method of quantifying bone resorption in aseptic loosening widely used is counting bone resorption pit formation in cortical bone slices (Pandey *et al*, 1996; Danks *et al*, 2000).

We set out to develop a simple *in vivo* model. The initial results of this part of the study demonstrated that sham surgery influenced the uptake of radioactive calcium. We were not given permission by the Home Office to operate on both femora in each mouse. Therefore, we had to use a set of animals to implant ceramic particles and another set to investigate the effects of sham surgery. The implantation of ceramic particles decreased the uptake of radioactive calcium compared to sham surgery at 4 weeks. This difference was statistically significant, however, the difference between the scintillation counts between the animals that had ceramic particles implanted and sham surgery was not clinically significant. We were expecting a minimum difference of 50 CPM/mg between the groups to be of clinical significance. There was no evidence in the literature at the time of

the study to base a significant difference level between the two groups being investigated and we arbitrarily set the difference of 50 CPM/mg. The difference of 50 CPM/mg was decided according to the following steps. It was decided with my supervisor and statistician to determine a meaningful difference after culling the first litter. We initially culled eight female mice so that we could determine the uptake of radioactive calcium in murine bone and have a guide to the value of scintillation counts. We expected that the initial count in the pregnant mice will distribute evenly to the number of offspring. We then decided to undertake the procedures and then cull the first litter and then estimate the significant difference between the two groups. However, there were only three mice in the first litter. We therefore decided to cull the second litter and then estimate a difference. It was decided in conjunction with the supervisor and statistician to set a difference of 50 CPM/mg after culling the first two litters. A sample size to detect a difference of 10, 50 and 100 CPM/mg between the groups would require 766, 31 and 8 animals in each group. We thought that the main reason for this small difference between the groups was the short period of time between the implantation of ceramic particles and culling of the animals. The possibility that a longer period of time will allow the inflammatory process and bone resorption to develop and allow us to detect a difference between the mice that underwent implantation of ceramic particles and sham surgery. Unfortunately, we were not able to detect a difference between the two animals groups at 10 weeks and our simple model failed to work. This model does show that the implantation of ceramic particles may cause bone resorption due to the fact that the scintillation

counts in the femora that underwent implantation of ceramic particles was less than those in femora that underwent sham surgery or control femora.

The possible reasons why the model did not work could be due to multiple factors. Factors that are known to be important in the development of osteolysis and aseptic loosening could be limiting factors in the development of this model. In this *in vivo* model, we used a large bolus of ceramic particles to cause osteolysis. Despite having a femur full of ceramic particles, we found no appreciable osteolytic effect in our model. This does not represent the true scenario of osteolysis were wear particles are generated continually at a variable rate rather than in a bolus. The mechanical loading and fluid pressure associated with implants were not represented in this model and could be a possible reason for the failure of the model. Experiments in a rabbit model using an exogenous derived fluid pressure has been shown to cause bone resorption (Van der Vis *et al*, 1998). Similar results have been obtained in a rat model (Skriptiz and Aspenberg, 2000). In a recent study by De Man *et al* (2005) using a rabbit model suggested that compression of a periprosthetic fibrous interface which induces fluid pressure and/or fluid flow can be an important cause of early loosening of THRs. Another possible reason for minimal osteolysis in our model could be the possibility that hyaluronic acid inhibited wear debris induced osteolysis.

However, we believe that the most important factor for the failure of development of this model is that we did not wait long enough after the implantation of the ceramic particles to detect osteolysis. Maybe we need to wait years for osteolysis to develop as is the case in THRs in humans.

Other animal models have been used in experimental studies with regards to the development of new materials and designs for implants. The dog has been the model of choice for pre-clinical trials of new implants for THR in man and the most common animal model (An and Friedman, 1998). Skurla *et al* (2005) showed that the incidence of aseptic loosening of the femoral component in dogs was much higher than human. They suggested that the clinical performance of canine THRs is more representative of that in young human patients and that the performance of implants differs between species. In addition to the canine THR model, the rabbit and rat have been used. The use of implants is more complex and more expensive. We hoped to create a simple model that could be reproduced by others using simple techniques and equipment. The purpose of a simple model was to investigate the efficacy of potential pharmaceutical agents *in vivo*, the mechanism of action, optimum dosage and the effect of combining pharmaceutical agents and eventually testing in human aseptic loosening. An optimal model to test pharmaceutical agents should allow rapid multiple testing plus be cheap and simple.

CHAPTER 4

URINARY MARKERS OF OSTEOLYSIS IN OUR ANIMAL MODEL

4.1 INTRODUCTION

Bone is a dynamic tissue consisting mainly of mineral, collagen, water and cells. The arrangement of the tissue is such that bone is organised optimally to resist loads imposed by functional activities. The mineral found in bone is hydroxyapatite. The second most abundant component of bone is collagen. Collagen provides bone with elasticity and flexibility and directs the organisation of the matrix. Approximately 90% of the organic matrix of bone is type I collagen, a triple helical protein. Type I collagen of bone is cross-linked at the N-terminal and C-terminal ends of the molecule which provide rigidity and strength. The N-telopeptide (NTx) molecule is specific to bone and its generation is mediated by osteoclasts on bone. NTx is found in urine as a stable end-product of degradation. Water accounts for 5-10% of the weight of bone tissue. Tissue hydration is needed both for nutrition and function of cells.

There are two histologically types of bone; compact and cancellous bone. Compact bone is solidly mineralised and is organised into Haversian systems. The Haversian systems consist of a canal containing a blood vessel surrounded by concentric lamellae. Whereas in cancellous bone, the matrix is organised into thin interconnecting spicules.

Bone is constantly undergoing a metabolic process called remodelling. This includes a degradation process, bone resorption, mediated by the action of osteoclasts and a building process, bone formation, mediated by osteoblasts. This process is necessary for normal development and maintenance of the skeleton. Osteoclasts resorb bone by acidification and dissolution of hydroxyapatite crystals

and proteolysis of the bone collagen matrix. The first process during osteoclastic bone resorption is mobilisation of hydroxyapatite crystals by digestion of their link to collagen via the non collagenous proteins. The low pH dissolves the hydroxyapatite crystals exposing the bone collagen matrix. Then the residual collagen fibres are digested by activated collagenase (Sims and Baron, 2000). Following this, collagen degradation products such as N-Telopeptide (NTx) and deoxypyridinoline (DPD) are released and it is possible to use these products as bone resorption markers.

The aim of this part of the study was to investigate whether urinary NTx and DPD can be used as *in vivo* bone resorption markers in our model. We also correlated the urinary markers NTx and DPD with the scintillation counts of femora that underwent implantation of ceramic particles.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

4.2.1.1 Animals

4.2.1.1.1 Operated Animals

The two sets of animals used in chapter 3 were utilised in this part of the study. As mentioned previously, there were two sets of animals that were utilised throughout the study. In each set of animals, the mouse either underwent the implantation of ceramic particles into the medullary canal of the femur or sham surgery which involved the injection of hyaluronic acid without ceramic particles into the medullary canal of the femur at the age of 10 weeks.

In the first set of animals, the mice were kept alive for 4 weeks after their surgical procedure and then culled.

In the second set of animals, the mice were kept alive for 10 weeks after their surgical procedure and then culled.

4.2.1.1.2 Control Animals

Control animals were utilised in this part of the project to compare urinary NTx and DPD concentrations to operated animals. The control animals used in this part of the study were not subjected to any surgical procedure. The control animals were approximately 18 weeks old and consisted of 24 animals (12 male and 12 female).

4.2.1.2 Urine Samples

Mice were placed in metabolic cages (Figure 4.1) the night before culling. Urine samples were collected the following morning and transferred into small

containers. Urine samples were spun at 10,000 revolutions per minute for 5 minutes so that contaminants would sediment and the supernatants were collected and frozen at -20°C. When required for biochemistry and ELISA, the supernatants were thawed for use.

4.2.1.3 ELISA kits

4.2.1.3.1 NTX Solutions required

Antibody Conjugate Concentrate – 0.4ml

Antibody Conjugate Diluent – 30ml

Buffered Substrate – 30ml

Chromogen Reagent – 0.9ml

Stopping Reagent – 25ml

Wash Concentrate – 125ml

4.2.1.3.2 DPD Solutions required

Enzyme Conjugate – three per kit

Assay Buffer – 55ml

Substrate Tablets – three per kit

Substrate Buffer – 30ml

Stopping Reagent – 15ml

Wash Concentrate – 55ml

4.2.1.4 Microwell plate reader

The microwell plate reader located in the Department of Respiratory Medicine at Glenfield Hospital, Leicester was used to read all the plates.

4.2.2 METHODS

4.2.2.1 Urine Creatitine

100µl of the thawed urine supernatant was used to calculate the urinary creatinine for each sample. This was performed at the Department of Biochemistry, Glenfield Hospital, Leicester using the Abbott Aeroset analyser (Abbott Lab Ltd., Berkshire, UK).

4.2.2.2 Urine NTx and DPD assays

4.2.2.2.1 NTx assay

1. The samples were analysed in batches. Each batch consisted of 96 samples.
2. Prior to analysis, the specimens and kit components were allowed to equilibrate to room temperature (18 – 28°C).
3. 25µL of urine samples, calibrators and control solutions were placed into the antigen coated 96 wells plate.
4. Urine samples, calibrators and controls were run in duplicate wells.
5. 200µL of working strength conjugate solution was placed into each well with a multichannel pipette.
6. A plate sealer was applied and the plate was swirled gently on a flat surface for 5 – 10 seconds.
7. The plate was incubated at room temperature (18 – 28°C) for 90 minutes.
8. At the end of the incubation period, the plate sealer was carefully removed and each well was washed with working strength wash solution. The wash

process was repeated five times. Wash volume was approximately 350µL wash solution per well.

9. 200µL of diluted chromogen reagent and buffered substrate was added to each well with a multichannel pipette.
10. A new plate sealer was applied and the plate was incubated at room temperature (18 – 28°C) for 15 minutes.
11. At the end of the incubation period, the plate sealer was carefully removed and 100µL of stopping reagent were added into the wells.
12. The plate was allowed to sit at room temperature for 5 minutes.
13. The absorbance values of the urine samples, calibrators and controls were read using a microwell plate reader within 30 minutes of adding the stopping reagent.

4.2.2.2.2 DPD assay

1. The samples were analysed in batches. Each batch consisted of 96 samples.
2. Prior to analysis, the specimens and kit components were allowed to equilibrate to room temperature (18 – 28°C).
3. 50µL of urine samples, calibrators and control solutions were diluted with 450µL of assay buffer provided by the manufacturer.
4. 50µL of diluted urine samples, calibrators and control solutions were added into the antigen coated 96 wells plate.
5. Urine samples, calibrators and controls were run in duplicate wells.
6. 100µL of working strength enzyme conjugate solution was placed into each well with a multichannel pipette.

7. A plate sealer was applied and the plate was incubated at 2 – 8°C for two hours. The incubation was carried out in the dark.
8. At the end of the incubation period, the plate sealer was carefully removed and each well was washed with working strength wash solution. The wash process was repeated three times. Wash volume was rough 250µL per wash.
9. Following the wash, 150µL of working substrate solution was added to each well.
10. A new plate sealer was applied and the plate was incubated at room temperature (20 – 28°C) for 60 minutes.
11. At the end of the incubation period, the plate sealer was carefully removed and 100µL of stopping reagent were added into the wells.
12. The plate was allowed to sit for a further 5 minutes at room temperature.
13. The absorbance values of the urine samples, calibrators and controls were read using a microwell plate reader within 15 minutes of adding the stopping reagent.

4.2.2.3 Urine NTx and DPD concentrations

4.2.2.3.1 Urine NTx concentration

The concentration values of urine samples and controls were determined from the calibration curve and values were expressed in nM BCE/mM creatinine (nanomoles Bone Collagen Equivalents per millimole creatinine per litre).

$$\text{Urine NTx concentration (nM BCE/mM)} = \frac{\text{Assay value (nM BCE)}}{\text{Urine creatinine (mM)}}$$

4.2.2.3.2 Urine DPD concentration

The concentration values of urine samples and controls were determined from the calibration curve and values were expressed in nM DPD/mM creatinine (nanomoles Deoxypyridinoline per millimole creatinine per litre).

$$\text{Urine DPD concentration (nM DPD/mM)} = \frac{\text{Assay value (nM DPD)}}{\text{Urine creatinine (mM)}}$$

4.2.2.4 Statistical analyses of data

Data from all three groups (ceramic particles, sham surgery and control) was analysed by Kruskal-Wallis test. Differences between the means of urinary NTx and DPD at 4 and 10 weeks was compared and determined by calculating the minimum significant difference ($p<0.05$) using Mann Whitney-U test. Pearson's correlation coefficient was used to correlate urinary NTx and DPD with scintillation counts of femora that underwent implantation of ceramic particles at 4 and 10 weeks.

4.3 RESULTS

4.3.1 Control set of animals urinary NTx and DPD concentrations

The following results are the urinary NTx and DPD results of 24 control animals.

Table 4.1

Mouse Number	NTx/Cr	DPD/Cr
1	37.44	11.31
2	117.04	16.10
3	78.916	15.33
4	32.86	8.19
5	59.27	15.66
6	101.49	17.71
7	47.52	13.59
8	65.17	7.79
9	192.55	18.28
10	63.99	13.14
11	64.36	14.13
12	96.15	7.99
13	73.69	9.91
14	98.34	18.07
15	72.15	9.49
16	71.99	12.72
17	178.38	17.23

18	50.81	10.96
19	94.35	15.65
20	143.86	13.52
21	44.18	16.15
22	102.04	13.32
23	55.46	11.81
24	103.11	10.09

Mean Urine NTx/Cr Concentration: 85.21

95% Confidence Interval for mean: 67.92 – 102.50

Standard Deviation: 40.95

Mean Urine DPD/Cr Concentration: 13.26

95% Confidence Interval for mean: 11.87 – 14.64

Standard Deviation: 3.28

4.3.2 First set of animals urinary NTx concentrations

4.3.2.1 NTx concentrations of animals with ceramic particles

The following results are the urinary NTx concentrations of the animals that had ceramic particles implanted into their femora. The results of 2 animals are not available due to inadequate urine samples being collected prior to culling.

Mouse number 43 sustained a fracture of the femur during the implantation of ceramic particles.

Table 4.2

Litter Number	Mouse Number	NTx/Cr
1	2	74.46
2	4	45.35
2	6	63.99
3	14	86.77
4	15	48.22
4	18	70.39
4	23	48.05
4	25	63.72
5	28	139.84
5	29	209.85
5	32	84.83
5	34	155.59
5	35	75.20
6	38	33.72
6	40	38.76
6	43	46.04
7	53	76.57
7	55	26.76
7	57	128.46
7	58	13.80
8	44	14.29

8	48	25.10
8	49	260.28
8	50	240.16

Mean Urine NTx/Cr Concentration: 86.26

95% Confidence Interval for mean: 57.16 – 115.36

Standard Deviation: 68.92

4.3.2.2 NTx concentrations of animals with sham surgery

The following results are the urinary NTx concentrations of the animals that underwent injection of hyaluronic acids (sham surgery). The results of 7 animals are not available due to inadequate urine samples being collected prior to culling. Mouse number 46 sustained a fracture of the femur during sham surgery.

Table 4.3

Litter Number	Mouse Number	NTx/Cr
2	5	48.27
2	7	30.14
4	17	54.81
4	24	76.89
5	26	64.53
5	27	65.40
5	30	158.88

5	31	214.99
5	33	167.88
6	36	65.72
6	37	57.07
6	39	76.57
6	42	71.88
7	54	12.87
7	56	134.04
7	59	84.99
7	60	33.50
8	46	23.60
8	47	13.28

Mean Urine NTx/Cr Concentration: 76.59

95% Confidence Interval for mean: 50.05 – 103.14

Standard Deviation: 55.07

4.3.3 Second set of animals urinary NTx concentrations

4.3.3.1 NTx concentrations of animals with ceramic particles

The following results are the urinary NTx concentrations of the animals that had ceramic particles implanted into their femora. Urine samples were collected from all animals.

Mouse number 36 sustained a fracture of the femur during the implantation of ceramic particles.

Table 4.4

Litter Number	Mouse Number	NTx/Cr
1	1	98.36
1	3	50.37
1	6	38.09
2	7	59.11
2	11	97.60
2	13	77.31
2	14	38.72
3	16	61.01
3	17	125.96
3	19	48.95
3	20	35.45
4	24	41.04
4	26	195.67
4	28	80.82
4	30	82.94
6	31	56.16
6	33	90.34
6	35	26.97
6	36	24.97
7	38	41.19
7	39	28.30

7	43	26.07
7	45	30.74
7	46	133.16

Mean Urine NTx/Cr Concentration: 66.22

95% Confidence Interval for mean: 48.59 – 83.84

Standard Deviation: 41.73

4.3.3.2 NTx concentrations of animals with sham surgery

The following results are the urinary NTx concentrations of the animals that underwent sham surgery. Urine samples were collected from all animals.

Table 4.5

Litter Number	Mouse Number	NTx/Cr
1	2	128.72
1	4	62.98
1	5	39.30
2	8	180.56
2	9	73.53
2	10	212.62
2	12	64.92
3	15	135.90
3	18	36.67

3	21	49.17
3	22	14.16
4	23	33.65
4	25	133.97
4	27	63.04
4	29	38.65
6	32	80.54
6	34	21.48
7	37	25.25
7	40	32.08
7	41	27.53
7	42	30.36
7	44	24.68

Mean Urine NTx/Cr Concentration: 68.63

95% Confidence Interval for mean: 44.19 – 93.06

Standard Deviation: 55.11

4.3.4 First set of animals urinary DPD concentrations

4.3.4.1 DPD concentrations of animals with ceramic particles

The following results are the urinary DPD concentrations of the animals that had ceramic particles implanted into their femora. The results of 3 animals are not available due to inadequate urine samples being collected prior to culling.

Mouse number 43 sustained a fracture of the femur during the implantation of ceramic particles.

Table 4.6

Litter Number	Mouse Number	DPD/Cr
1	2	7.32
2	4	6.49
3	9	13.39
3	14	4.01
4	15	2.55
4	23	10.09
4	25	5.60
5	29	13.73
5	32	6.16
5	34	2.86
5	35	5.94
6	38	20.68
6	40	21.16
7	55	23.58
7	53	9.37
6	43	15.87
7	57	11.24
7	58	9.79

8	44	9.612
8	45	12.74
8	48	11.70
8	49	9.88
8	50	12.95

Mean Urine NTx/Cr Concentration: 10.72

95% Confidence Interval for mean: 8.28 – 13.17

Standard Deviation: 5.66

4.3.4.2 DPD concentrations of animals with sham surgery

The following results are the urinary DPD concentrations of the animals that underwent sham surgery. The results of 9 animals are not available due to inadequate urine samples being collected prior to culling.

Mouse number 46 sustained a fracture of the femur during sham surgery.

Table 4.7

Litter Number	Mouse Number	DPD/Cr
2	5	4.92
2	7	2.89
3	13	5.09
4	19	2.03
5	26	8.89

5	27	13.95
5	30	10.60
5	33	13.87
6	36	15.58
6	37	6.82
6	39	10.58
6	42	14.29
7	56	7.60
7	60	19.84
8	46	6.95
8	47	16.79
8	52	25.39

Mean Urine NTx/Cr Concentration: 10.95

95% Confidence Interval for mean: 7.71 – 14.19

Standard Deviation: 6.30

4.3.5 Second set of animals urinary DPD concentrations

4.3.5.1 DPD concentrations of animals with ceramic particles

The following results are the urinary DPD concentrations of the animals that had ceramic particles implanted into their femora. Urine samples were collected from all animals.

Mouse number 36 sustained a fracture of the femur during the implantation of ceramic particles.

Table 4.8

Litter Number	Mouse Number	DPD/Cr
1	1	7.48
1	3	7.22
1	6	5.14
2	7	4.29
2	11	3.29
2	13	9.49
2	14	8.83
3	16	4.36
3	17	5.82
3	19	7.62
3	20	10.73
4	24	3.87
4	26	3.94
4	28	9.70
4	30	8.72
6	31	3.91
6	33	6.69
6	35	5.67
6	36	2.69
7	38	10.53
7	39	9.94

7	43	10.52
7	45	10.66
7	46	10.29

Mean Urine NTx/Cr Concentration: 7.14

95% Confidence Interval for mean: 5.98 – 8.30

Standard Deviation: 2.75

4.3.5.2 DPD concentrations of animals with sham surgery

The following results are the urinary DPD concentrations of the animals that underwent sham surgery. Urine samples were collected from all animals.

Table 4.9

Litter Number	Mouse Number	DPD/Cr
1	2	6.92
1	4	10.23
1	5	7.72
2	8	5.52
2	9	5.37
2	10	6.13
2	12	3.87
3	15	5.06
3	18	3.40

3	21	4.34
3	22	1.79
4	23	4.86
4	25	5.71
4	27	9.15
4	29	3.39
6	32	6.39
6	34	3.39
7	37	3.64
7	40	2.59
7	41	11.49
7	42	8.66
7	44	2.16

Mean Urine NTx/Cr Concentration: 5.54

95% Confidence Interval for mean: 4.37 – 6.70

Standard Deviation: 2.62

4.3.6 Summary of urinary marker results of first set of animals

Urinary markers of both groups – mean (standard deviation) at 4 weeks

Table 4.10

Group	Number of animals	Urinary NTx	Urinary DPD
Ceramic particles	25	130.29 (66.20)	10.73 (5.66)
Sham surgery	19	76.60 (55.07)	10.95 (6.30)
		p = 0.864	p = 0.887

4.3.7 Summary of urinary marker results of second set of animals

Urinary markers of both groups – mean (standard deviation) at 10 weeks

Table 4.11

Group	Number of animals	Urinary NTx	Urinary DPD
Ceramic particles	27	72.45 (44.42)	7.26 (2.75)
Sham surgery	25	67.98 (53.21)	5.68 (2.68)
		p = 0.613	p = 0.045

4.3.8 Statistical analysis of urinary NTx and DPD at 4 weeks

4.3.8.1 Urinary NTx analysis

The urinary NTx concentrations between animals at 4 weeks that underwent the implantation of ceramic particles into their femora compared to those who underwent sham surgery was not statistically significant (p=0.864).

The comparison of urinary NTx of all three groups at 4 weeks (ceramic particles, sham surgery and control) was also not statistically significant (p=0.834).

The effect of a fracture of the femur that occurred in 2 animals on urinary NTx was not statistically significant (p=0.236).

4.3.8.2 Urinary DPD analysis

The urinary DPD concentrations between animals at 4 weeks that underwent the implantation of ceramic particles into their femora compared to those who underwent sham surgery was not statistically significant (p=0.837).

The comparison of urinary DPD of all three groups at 4 weeks (ceramic particles, sham surgery and control) was also not statistically significant (p=0.188).

The effect of a fracture of the femur that occurred in 2 animals on urinary DPD was not statistically significant (p=0.904).

4.3.9 Statistical analysis of urinary NTx and DPD at 10 weeks

4.3.9.1 Urinary NTx analysis

The urinary NTx concentrations between animals at 10 weeks that underwent the implantation of ceramic particles into their femora compared to those who underwent sham surgery was not statistically significant (p=0.613).

The comparison of urinary NTx of all three groups at 10 weeks (ceramic particles, sham surgery and control) was also not statistically significant (p=0.309).

The effect of a fracture of the femur that occurred in 1 animal on urinary NTx was not statistically significant (p=0.294).

4.3.9.2 Urinary DPD analysis

The urinary DPD concentrations between animals at 10 weeks that underwent the implantation of ceramic particles into their femora compared to those who underwent sham surgery was statistically significant ($p=0.045$).

The comparison of urinary DPD of all three groups at 10 weeks (ceramic particles, sham surgery and control) was also statistically significant ($p<0.001$).

The effect of a fracture of the femur that occurred in 1 animal on urinary DPD was not statistically significant ($p=0.052$). Surprisingly, the urinary DPD levels of the control animals were higher than the mice that underwent implantation of ceramic particles and injection of hyaluronic acid. This could possibly be explained by an error during the measurement of urinary DPD levels using the ELISA kits. The urinary DPD levels of the control animals were performed alone and in batches. Whereas, the urinary DPD levels of the animals that underwent implantation of ceramic particles and injection of hyaluronic acid were performed together in batches. We were only interested in the urinary markers of the two groups and the control urinary markers were used as a guide only. Also it is unlikely there was an error with the measurement of urinary creatinine levels since the urinary creatinine levels were all performed at the same time interval.

4.3.10 Statistical analysis of urinary markers between week 4 and 10

The concentration of urinary NTx in animals that were culled at 4 weeks compared to those that were culled at 10 weeks was not statistically significant ($p=0.732$). However, the urinary DPD concentrations differed between the two sets of animals and this was statistically significant ($p=0.002$).

4.3.11 Box plot comparing NTx and DPD at 4 weeks for all groups

Figure 4.1

Box plot of urinary NTx at 4 weeks

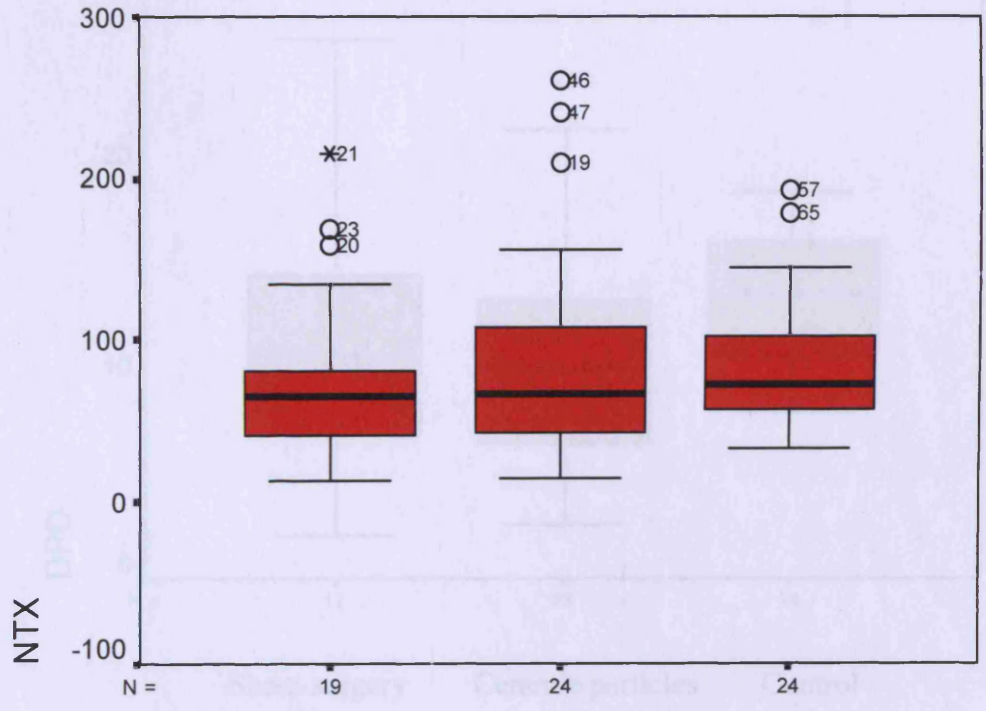
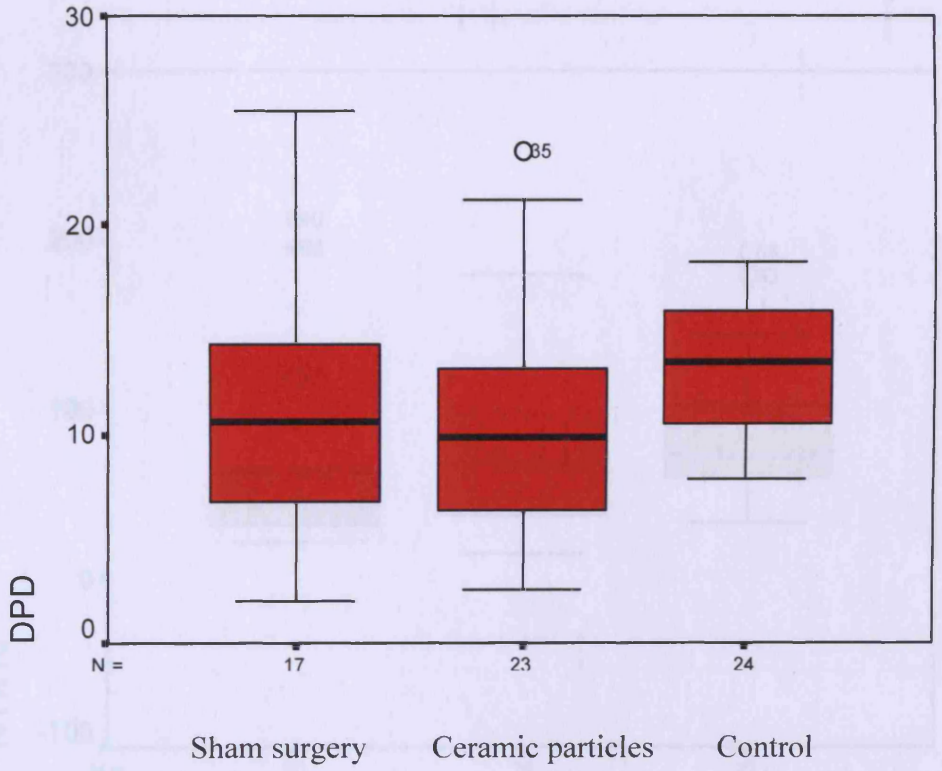


Figure 4.2

Box plot of urinary DPD at 4 weeks



4.3.12 Box plot comparing NTx and DPD at 10 weeks for all groups

Figure 4.3

Box plot of urinary NTx at 10 weeks

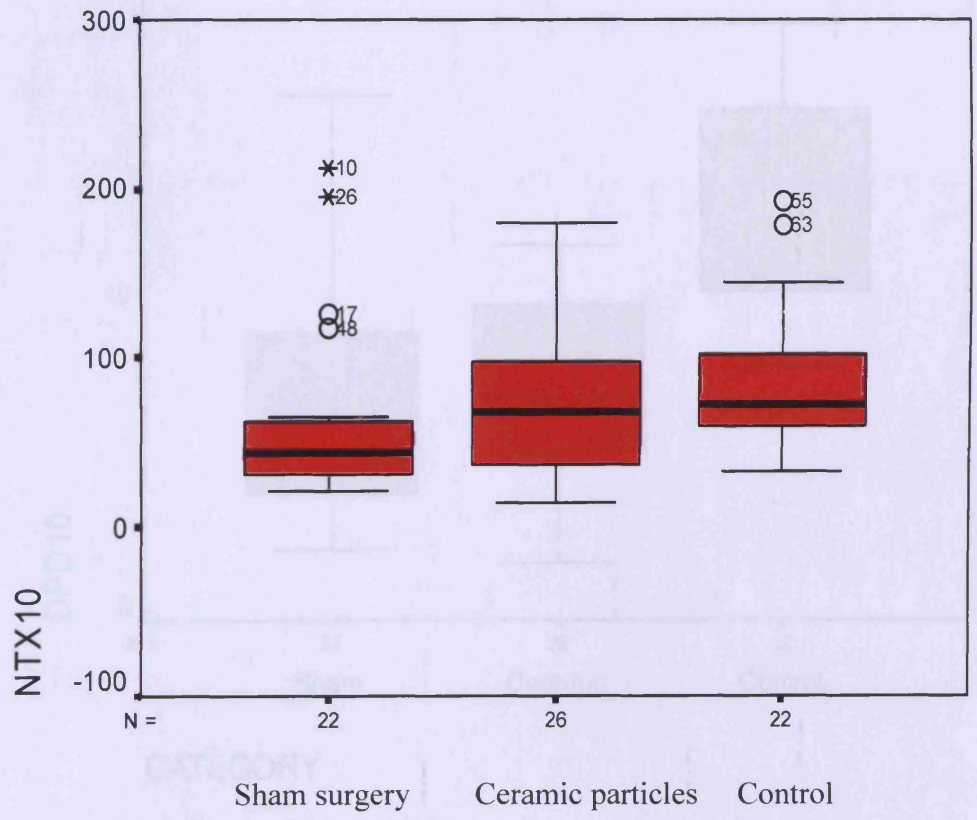
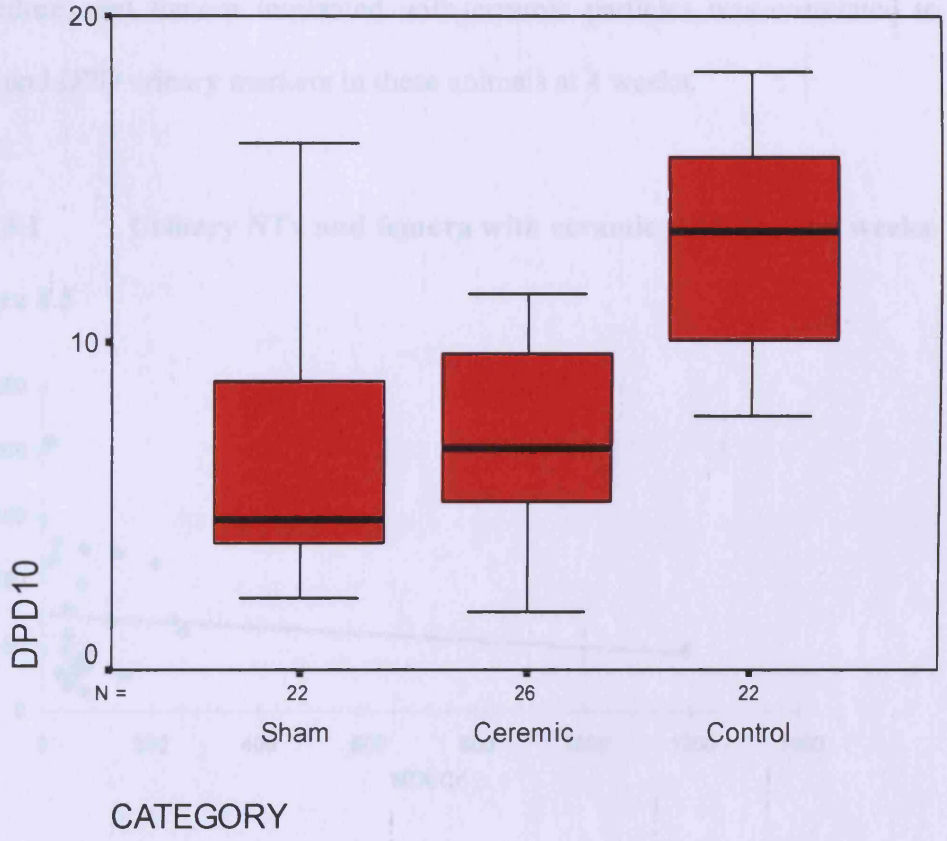


Figure 4.4

Box plot of urinary DPD at 10 weeks

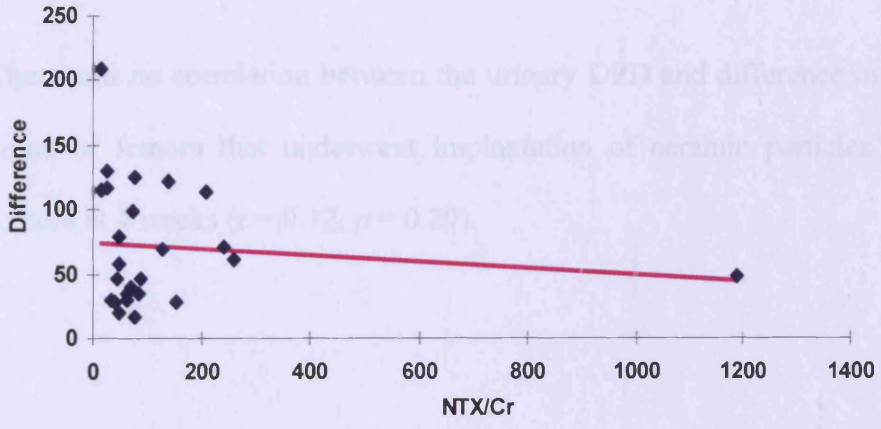


4.3.13 Correlation of urinary markers and femora with ceramic particles at 4 weeks

The difference in scintillation count between the control femora (no surgical procedure) and femora implanted with ceramic particles was correlated to the NTx and DPD urinary markers in these animals at 4 weeks.

4.3.13.1 Urinary NTx and femora with ceramic particles at 4 weeks

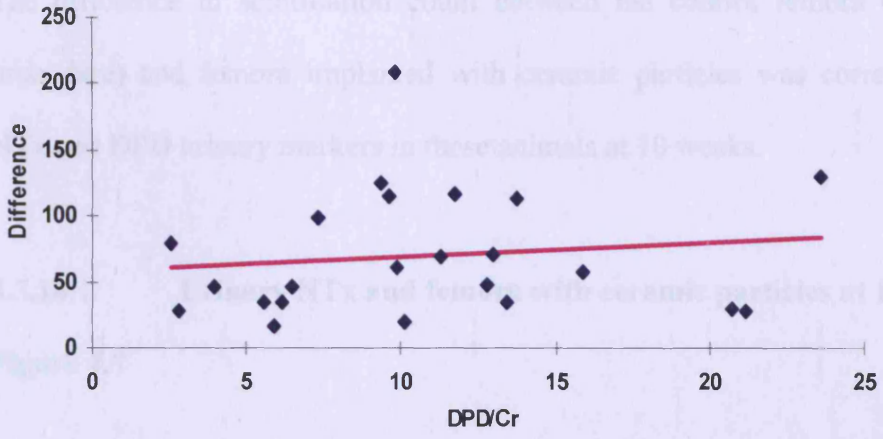
Figure 4.5



There was no correlation between the urinary NTx and difference in scintillation count of femora that underwent implantation of ceramic particles and control femora at 4 weeks ($r = -0.12$, $p = 0.72$).

4.3.13.2 Urinary DPD and femora with ceramic particles at 4 weeks

Figure 4.6



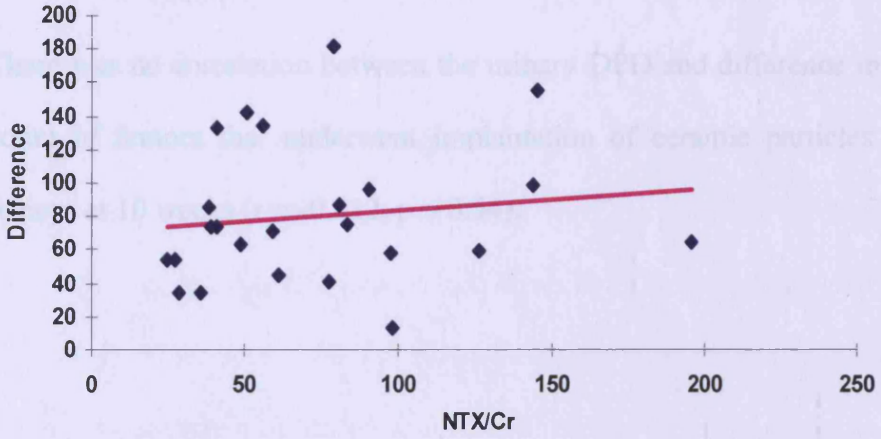
There was no correlation between the urinary DPD and difference in scintillation count of femora that underwent implantation of ceramic particles and control femora at 4 weeks ($r = 0.12$, $p = 0.29$).

4.3.14 Correlation of urinary markers and femora with ceramic particles at 10 weeks

The difference in scintillation count between the control femora (no surgical procedure) and femora implanted with ceramic particles was correlated to the NTx and DPD urinary markers in these animals at 10 weeks.

4.3.14.1 Urinary NTx and femora with ceramic particles at 10 weeks

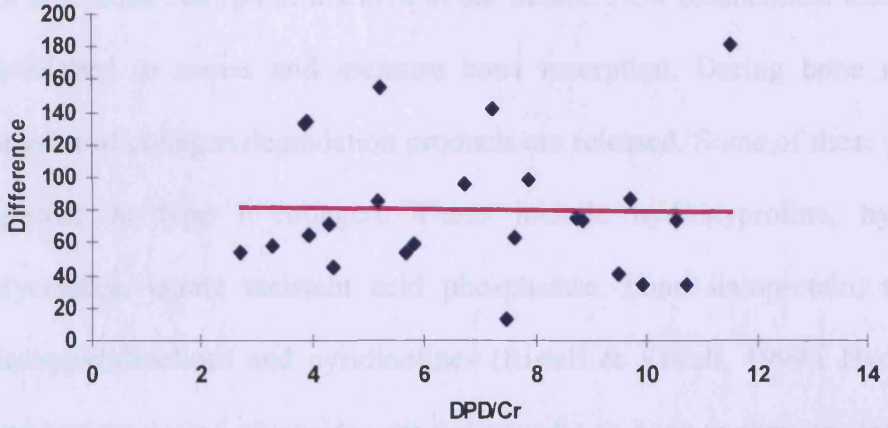
Figure 4.7



There was no correlation between the urinary NTx and difference in scintillation count of femora that underwent implantation of ceramic particles and control femora at 10 weeks ($r = 0.14$, $p = 0.26$).

4.3.14.2 Urinary DPD and femora with ceramic particles at 10 weeks

Figure 4.8



There was no correlation between the urinary DPD and difference in scintillation count of femora that underwent implantation of ceramic particles and control femora at 10 weeks ($r = -0.012$, $p = 0.54$).

4.4 DISCUSSION

The aim of this part of the study was investigate the possibility of using NTx and DPD as bone resorption markers in our model. New biochemical tests have been developed to assess and measure bone resorption. During bone resorption a number of collagen degradation products are released. Some of these products are specific to type I collagen. These include hydroxyproline, hydroxylysine glycosides, tatrate resistant acid phosphatase, bone sialoprotein, telopeptides, deoxypyridinolines and pyridinolines (Risteli & Risteli, 1999). Hydroxyproline and hydroxylysine glycosides are not specific to bone as they are found in other connective tissue. Some of the collagen degradation products in the urine, particularly the cross-linked telopeptides (NTx) and pyridinolones (DPD) have the highest specificity to bone resorption activity. NTx appears to be the most specific and responsive marker of systemic osteoclast activity (Schneider *et al*, 1997). The biochemical indices of bone turnover provide complementary information that can aid in predicting risk of future bone loss. In addition, bone responsive markers can be used to monitor effectiveness of therapy.

Urinary NTx has been found to be elevated in patients with aseptic loosening. Schneider *et al* (1997) and Antoniou *et al* (2000) found elevated urinary levels of NTx in their clinical studies of aseptic loosening. There is a need to identify non-invasive surrogate markers that could indicate the onset of aseptic loosening and lead to earlier diagnosis. Currently, no loosening-specific molecule or marker has yet been identified. Apone *et al* (1997) showed that NTx was generated when osteoclasts were cultured with human bone fragments and the NTx concentration

correlated strongly with the resorbed area on dentin slices. Scheven *et al* (1997) also showed that NTx when used as a bone resorption marker to monitor osteoclast activity correlated strongly with tartrate resistant acid phosphatase (TRAP) activity which is a marker of bone resorbing ability of cells. More recently, Savarino *et al* (2005) analysed systemic NTx for the assessment of osteolysis. Twenty one patients with radiological evidence of loosening, 18 patients with well-fixed prosthesis and 17 patients at the time of primary replacement for osteoarthritis were recruited. NTx was significantly increased only in the patients with loosening suggesting collagen degradation products depended on altered bone turnover induced by the implant. The other two groups of patients have similar NTx levels to healthy control subjects.

Unfortunately, our present model was unable to detect a difference in the urinary NTx concentration between animals that underwent the implantation of ceramic particles into their medullary canal compared to sham surgery. The urinary NTx concentration was not statistically different between the two groups even when keeping the ceramic particles in the medullary canal from 4 to 10 weeks. This can be attributed to the absence of osteolysis in our model. Our results also showed no difference between the two treatment groups (ceramic particles and sham surgery) and control urinary NTx concentrations at 4 and 10 weeks.

DPD has also been used extensively in other research studies as a bone resorption marker (Robins *et al*, 1986; Demers & Kleerkoper, 1995). DPD is released during bone resorption and is expected to rise if bone resorption is present. In this part of

the study we have shown some promising results with the urinary DPD concentrations. We were able to show a difference between the urinary DPD concentrations between animals that had ceramic particles implanted into their femora compared to those who underwent sham surgery. This difference was only significant at 10 weeks after implantation of ceramic particles. The urinary DPD concentrations were higher in the animals that had the ceramic particles compared to sham surgery but the concentrations were higher in the control animals.

Our results show that urinary DPD concentration may be a more sensitive marker in this model as concentrations were higher and statistically significant when compared to urinary NTx concentrations. We believe that using NTx and DPD as bone resorption markers can avoid the use of radioactive materials and it is cheaper. Furthermore, the potential treatment of osteolysis with pharmaceutical agents could be monitored by directly quantifying the concentrations of these bone markers.

In this *in vivo* model of wear debris induced osteolysis, most of the tested urinary markers of bone resorption were negative. This could be explained by the lack of bone resorption activity in the model and that osteolysis by ceramic particles takes at least 10 weeks to start or that the urinary bone markers simply did not reach a detectable level of significance if bone resorption was present. The urinary DPD results were higher at 10 weeks and if we had waited longer, we might have seen more promising results. This is consistent with the late appearance of osteolysis clinically often after many years. We were also unable to detect a correlation

between the tested urinary markers and difference in scintillation count of the femora that underwent implantation of ceramic particles and control femora. This could due to the lack of osteolysis by ceramic particles in the femora with variable scintillation counts and/or the low and variable levels of urinary markers of bone resorption in the model.

Figure 4.9

Metabolic Cage for urine collection

CHAPTER 5

MATRIX METALLOPROTEINASE-9 IN OUR ANIMAL MODEL

5.1 INTRODUCTION

Matrix Metalloproteinases (MMPs) are a family of at least 16 zinc endopeptidases secreted by cells. These enzymes are capable of degrading almost all components of the extracellular matrix. MMPs have a number of common properties: (1) they contain common sequences of amino acids; (2) they are secreted as inactive proenzymes; (3) activation is achieved proteolytically and is accompanied by a decrease in molecular weight; (4) they contain zinc at the active centre; and (5) the active enzymes are inhibited by tissue inhibitors of metalloproteinases (TIMPs). These enzymes act at neutral pH and require calcium for activity.

MMPs are divided into four main groups. These are called stromeolysins, collagenases, gelatinases and membrane-type metalloproteinases. MMPs take part in the extracellular matrix degradation in both physiological tissue remodelling and pathological conditions. One of the important roles of MMPs in matrix degradation in physiologic tissue is bone turnover. MMPs are also implicated in pathological destruction of cartilage and bone tissue in several arthritides.

MMPs are controlled by (1) the stimulation of various cytokines and growth factors that regulate synthesis and secretion; (2) the activation of proMMPs; and (3) the inhibition of active MMPs. Various inflammatory cytokines such as IL-1 and TNF-α stimulate the secretion of MMPs. Growth factors such as PDGF, FGF-2 and EGF can up regulate MMPs (Shingleton *et al*, 1996). Thus, the control of MMPs is complex with cell and matrix interactions modifying responses. All the MMPs are produced in a proenzyme form that requires the removal of a

10kDa N-terminal fragment to activate the enzyme. Many active MMPs can activate other pro-MMPs. All active MMPs are inhibited by TIMPs and all connective tissues contain members of the TIMP family (Tagaki, 1996; Willenbrock *et al*, 1995). There are currently four known TIMPs and they operate with different inhibition efficiencies against the different MMPs. The TIMPs play an important role in controlling connective tissue breakdown by blocking the action of activated MMPs and preventing activation of the proenzymes.

Extracellular matrix degradation and periprosthetic connective tissue remodelling around implants have been regarded as major biologic events in the loosening of total hip prostheses. The contribution of MMPs and their inhibitors is also thought to be important in this process (Ishiguro *et al*, 1996; Santavirta *et al*, 1993; Takagi *et al*, 1998). Previous studies have suggested that the imbalance of MMPs and TIMPs activity may contribute to prosthetic loosening (Takagi *et al*, 1994; Yokohama *et al*, 1995; Takei *et al*, 2000).

The aim of this study was to demonstrate the presence of MMP-9 in our animal model of wear debris induced osteolysis and whether there was a quantitative change in its presence over time. A further aim of this part of the study was determine whether there was an increase in the macrophage and osteoclast response after implantation of ceramic particles into the murine femora compared to the non operated femora.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

5.2.1.1 Animals

5.2.1.1.1 First set of animals

Eight mice all from the same litter were used in this part of the study. The animals were equally randomly allocated to either implantation of ceramic particles into their femora or sham surgery with injection of hyalrounic acid. The other femur was used as control and did not undergo any surgical procedure. All animals were 10 weeks old at the time of surgery and culled 4 weeks after surgery.

5.2.1.1.2 Second set of animals

Six mice all from the same litter were used in this part of the study. The animals were equally randomly allocated to either implantation of ceramic particles into their femora or sham surgery with injection of hyaluronic acid. The other femur was used as control and did not undergo any surgical procedure. All animals were 10 weeks old at the time of surgery and culled 10 weeks after surgery.

5.2.1.2 Bone

Both femora were collected from each mouse as soon as the animal was culled. The operated and control femora were labelled to avoid mistakes.

5.2.1.3 Positive controls

Positive control slides containing MMP-9 was provided by Professor R Walker from the Department of Pathology, Glenfield Hospital, Leicester. The slides

consisted of infiltrating lobular carcinoma of the breast with tumour cells expressing MMP-9.

5.2.1.4 MMP-9 antibody

Rabbit anti-mouse MMP-9 full length polyclonal antibody was purchased from Chemicon Europe (Hamphshire, UK).

The kit contained 100µg (1 mg/mL) of purified, intact mouse full length MMP-9.

5.2.1.5 Envision kit

The DAKO EnVision™ Plus HRP kit for use with Rabbit Primary Antibodies was purchased from DakoCytomation (Cambridgeshire, UK).

The kit contained the following reagents:

0.03% hydrogen peroxide – 15mL

Peroxide labelled polymer goat anti-rabbit immunoglobulin – 15mL

3,3'-diaminobenzidine (DAB+) substrate buffer solution – 18mL

DAB+ chromogen solution – 1mL

5.2.1.6 Microscope

All tissue sections were analysed using light microscopy (Olympus BH2-RFCA).

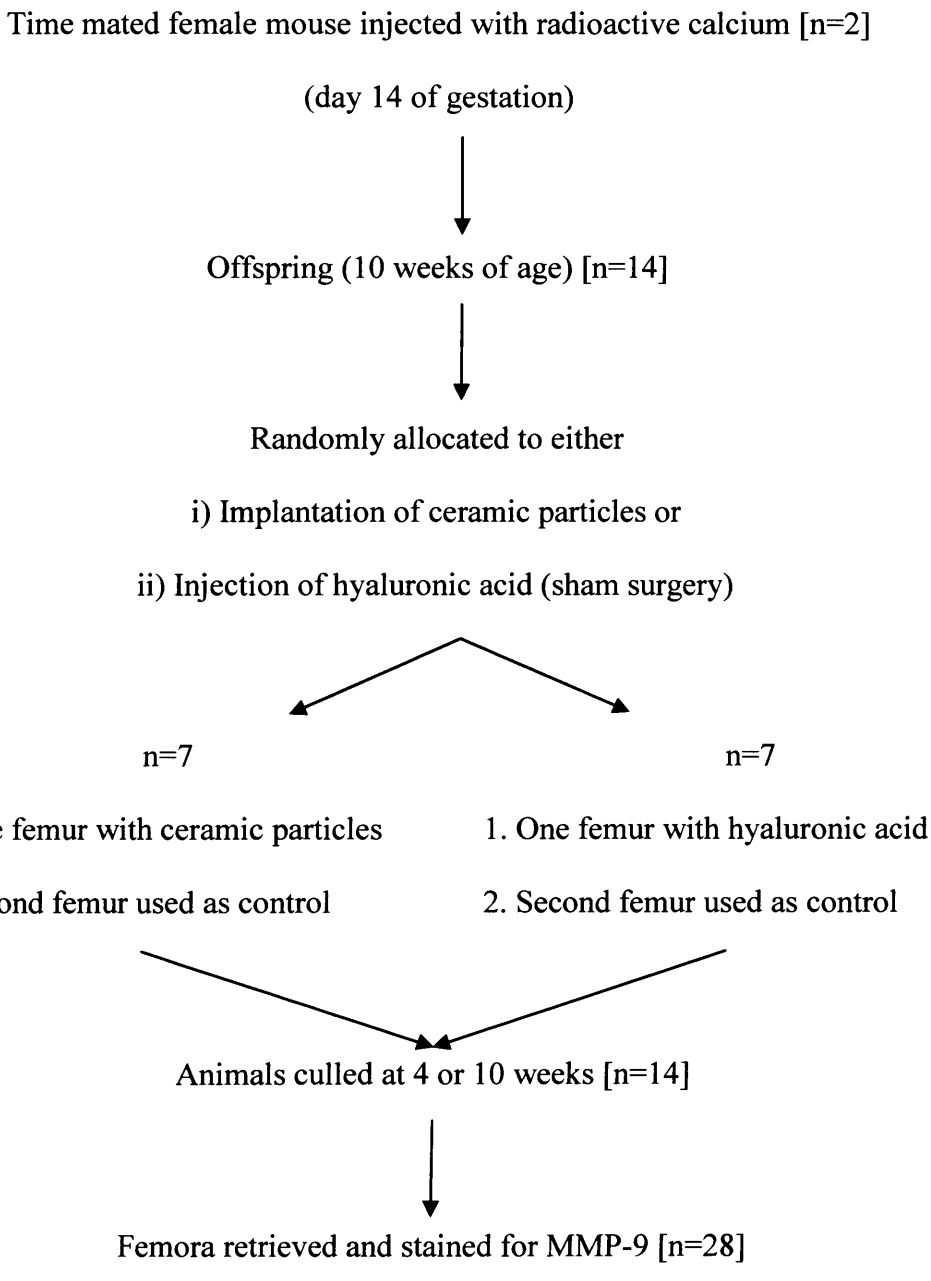
5.2.2 METHODS

5.2.2.1 Flow Diagram

The following flow diagram displays the steps undertaken in this part of the study.

Figure 5.1

Outline of the study for MMP staining



5.2.2.2 Particles and sham surgery

The animals underwent the same surgical procedure as outlined in chapter 3 either having the implantation of ceramic particles or injection of hyaluronic acid into their femoral medullary canal.

The 2 different sets of animals were culled at different time intervals. The first set was culled after 4 weeks of surgery whereas, the second was culled after 10 weeks of surgery.

5.2.2.3 Bone retrieval

Animals were culled using a schedule 1 procedure. Femora were retrieved soon after culling through an incision made along its length. All soft tissue was dissected off the femora. The femora were then cut using a scalpel (number 15 blade) at the neck of the femur and supracondylar region.

All femora after retrieval were placed in 10% formalin and then transferred to Glenfield Hospital, Leicester.

All femora were place in 10% formic acid overnight for decalcification.

5.2.2.4 Immunohistochemistry staining

The immunohistochemistry staining was adapted from Lunsuwanont *et al* (2002) and the same procedure was used for MMP-9.

Paraffin sections of breast tissue as described in 5.2.1.3 were used as a positive control for MMP-9 immunohistochemistry procedures. The procedure was carried out on 5μm sections placed on vector bonded slides (described in section 2.2.5).

The sections were deparaffinated in xylene for 5 minutes and were transferred through graded alcohols for 2 minutes and then placed in running water for 5 minutes. Slides were then rinsed with Tris-Buffered Saline (0.15M NaCl in 0.05M Tris/HCL-Buffer, pH 7.6; TBS). Endogenous peroxidases were blocked by treating the sections with peroxidase block (provided in kit) for 5 minutes. Slides were then rinsed in cold TBS twice for 2 minutes. Sections were then treated with a pre-blocking agent (200μL/slide) for 20 minutes by adding 1.5% normal goat serum in TBS. The sections were then rinsed in TBS for 5 minutes. Each section then had 200μL/slide of primary polyclonal antibody (MMP-9) added and TBS for the negative control with no primary antibody. The sections were then rinsed in cold TBS twice for 5 minutes. All sections were then incubated overnight at $4^{o}C$.

After incubation, the sections were treated with the Horse Radish Peroxidase (HRP) labelled polymer which is conjugated with secondary antibodies (DAKO EnVision™) for 30 minutes. The sections were then rinsed in TBS twice for 5 minutes. The sections were then stained with diaminobenzidine (DAB) for 2 to 7 minutes. Sections were then rinsed in running water for 5 minutes and counterstained using haematoxylin for 30 seconds. Sections were then rinsed again in water for 2 minutes and then dehydrated through graded alcohols and transferred to fresh xylene. Sections were then mounted using Dibutylphthalate Xylene (DPX) mountant.

Each set of femora slides were stained with two negative controls (Figure 5.1) and two positive controls (Figure 5.2) using the breast lobular carcinoma tissue to confirm that the immunohistochemistry staining was working properly.

For each femora, several sections were cut. Some of the cuts were used for immunohistochemistry staining with MMP-9 (Figure 5.4) whereas the others were used as negative controls (Figure 5.3).

5.2.2.5 Grading system used in staining

Several sections from each femora were taken. Tissue sections were analysed using light microscopy to determine the histological staining of MMP-9. Two independent reviewers examined all slides.

The femora were quantitatively graded for the amount of MMP-9 staining by examining macrophages and osteoclasts. The cells were identified by looking at cell size and cytoplasmic colour. The amount of staining per slide was calculated by dividing the number of macrophages and osteoclasts stained on the slide by the total number of macrophages and osteoclasts on the negative control slide.

Percentage of staining of MMP-9 =

$$\frac{\text{Number of macrophages/osteoclasts stained for MMP-9}}{\text{Total number of macrophages/osteoclasts on negative control}}$$

Each slide was examined by each reviewer twice. The average of the percentage of staining was then calculated. Therefore, each slide had two scores after being examined by both reviewers. All slides were examined under x 100 magnification.

5.2.2.6 Grading of macrophage and osteoclast response

The number of macrophages and osteoclasts on the slides of the femora that underwent implantation of ceramic particles were compared to the number of macrophages and osteoclasts on the slides of the femora that did not undergo any surgical procedure. The comparisons were made at both 4 and 10 weeks after culling of the mice.

5.2 RESULTS

5.3.1 MMP-9 Staining

5.3.1.1 First set of animals

Table 5.1

Animal	Ceramic Particles	Sham Surgery	Control
1	55%		0%
	58%		
2		18%	0%
		22%	
3	48%		13%
	30%		
4		14%	8%
		14%	
5	48%		2%
	43%		
6	79%		0%
	46%		
7		13%	13%
		7%	
8		9%	0%
		10%	

Comparing the staining of MMP-9 between the femora that underwent implantation of ceramic particles and those that underwent injection of hyaluronic acid (sham surgery) at 4 weeks showed a statistical difference ($p < 0.001$). The results show more staining for MMP-9 with the implantation of ceramic particles compared to sham surgery at 4 weeks.

5.3.1.2 Second set of animals

Table 5.2

Animal	Ceramic Particles	Sham Surgery	Control
1		26%	0%
		24%	
2	38%		2%
	32%		
3	47%		6%
	40%		
4	34%		8%
	44%		
5		14%	3%
		21%	
6		24%	3%
		27%	

Comparing the staining of MMP-9 between the femora that underwent implantation of ceramic particles and those that underwent injection of hyaluronic acid (sham surgery) at 10 weeks showed a statistical difference ($p = 0.004$). The results show more staining of MMP-9 with the implantation of ceramic particles compared to sham surgery at 10 weeks.

In support of the above results, comparison of all three groups at week 4 and 10 did show statistical significance ($p < 0.001$).

To quantify an increase in MMP-9 staining with the implantation of ceramic particles between week 4 and 10, a comparison was made. Results failed to show any statistically difference in the amount of MMP-9 staining ($p = 0.053$).

5.3.1.3 Summary of MMP-9 staining

The following table is a summary of the MMP staining in all femora in the first and second sets of animals.

Table 5.3

The overall amount of MMP-9 staining – mean (standard deviation)

Group	Number of animals	Ceramic particles	Sham surgery	Control femora
First set (4 weeks)	8	51% (0.14)	13% (0.05)	5% (0.06)
Second set (10 weeks)	6	39% (0.06)	23% (0.05)	4% (0.03)

5.3.2 Macrophage and osteoclast response

5.3.2.1 First set of animals

Table 5.4

Mean number of macrophage and osteoclast per slide

Ceramic particles	Sham surgery	Control
118	42	22
84	37	33
97	30	18
88	48	20
71	34	13
87	22	17
94	20	16
60	22	10

We were able to detect a statistically significant difference between the number of macrophages and osteoclasts in the femora that underwent implantation of ceramic particles compared to the control femora at 4 weeks ($p < 0.001$).

We were also able to detect a statistically significant difference between the number of macrophages and osteoclasts in the femora that underwent injection of hyaluronic acid compared to the control femora at 4 weeks ($p = 0.007$).

5.3.2.2 Second set of animals

Table 5.5

Mean number of macrophage and osteoclast per slide

Ceramic particles	Sham surgery	Control
90	41	60
131	39	18
94	36	52
81	28	29
73	45	38
85	48	30

We were able to detect a statistically significant difference between the number of macrophages and osteoclasts in the femora that underwent implantation of ceramic particles compared to the control femora at 10 weeks ($p = 0.004$).

We were not able to detect a statistical difference between the number of macrophages and osteoclasts in the femora that underwent injection of hyaluronic compared to the control femora at 10 weeks ($p = 0.75$).

5.3.2.3 Summary of macrophage and osteoclast response

The following table is a summary of the mean number of macrophages and osteoclasts per slide in all femora in the first and second sets of animals.

Table 5.6

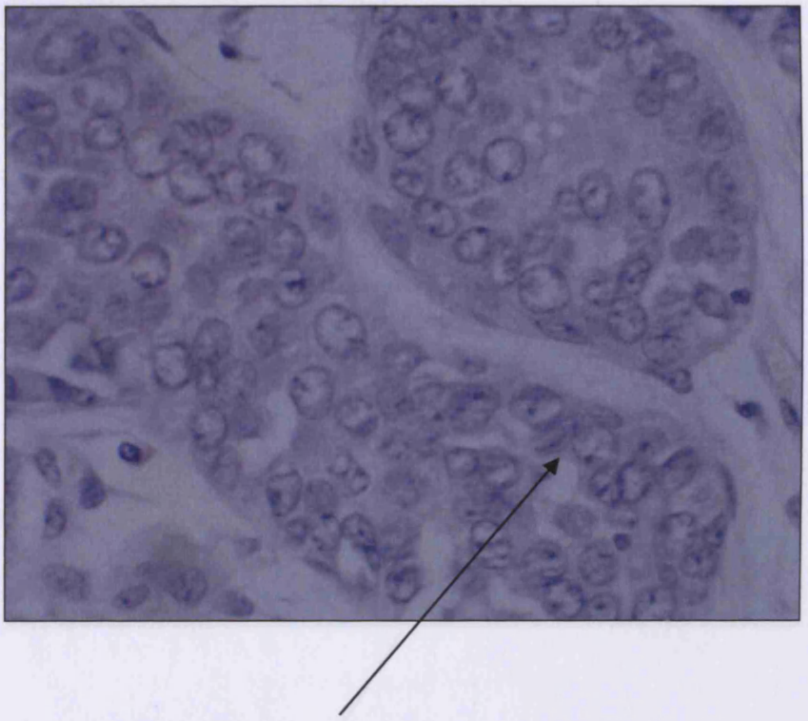
Summary of macrophage and osteoclast response in all femora – mean (standard deviation)

Group	Number of animals	Ceramic particles	Sham surgery	Control femora
First set (4 weeks)	8	87 (17)	32 (10)	19 (7)
Second set (10 weeks)	6	92 (20)	40 (7)	39 (16)

We did not detect a statistical difference between the number of macrophages and osteoclasts in the femora that underwent implantation of ceramic particles at weeks 4 and 10 ($p = 0.85$).

Figure 5.2

Section of breast lobular carcinoma with no primary antibody (Negative Control); × 100 magnification.



Lobular carcinoma cells with no staining (negative control)

Figure 5.3

Section of breast lobular carcinoma with primary antibody (Positive Control); × 100 magnification.

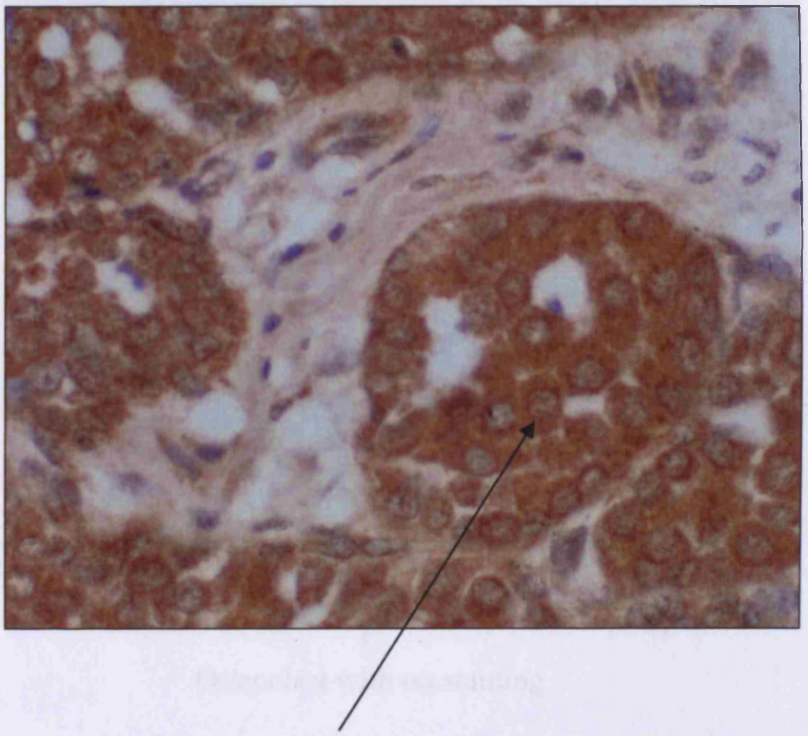


Figure 5.4

Section of murine femur with no primary antibody; × 100 magnification.

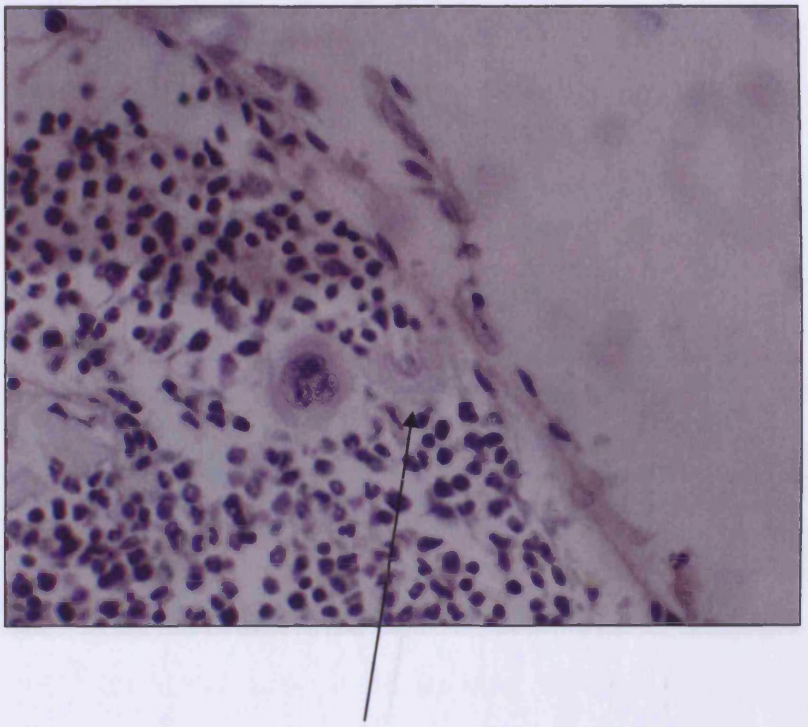
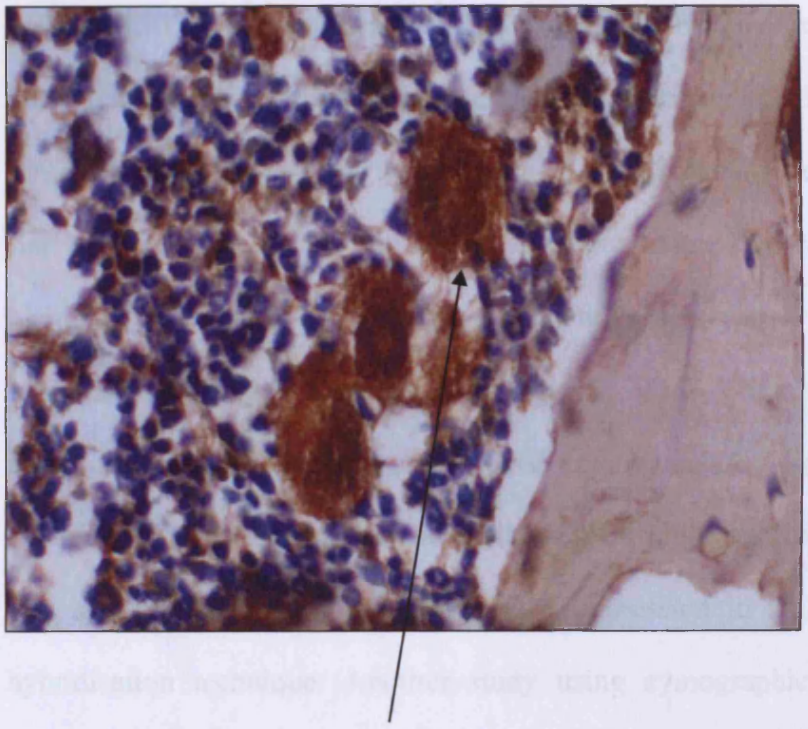


Figure 5.5

Section of murine femur with primary antibody; × 100 magnification.



Osteoclast with MMP-9 staining

5.4 DISCUSSION

MMPs are widely and differentially distributed throughout developing human bone. MMP-9 is synthesised and released by neutrophils, osteoclasts, macrophages and fibroblasts in granuloma. (Hibbs *et al*, 1985; Lorenzo *et* al, 1992; Mainardi *et* al, 1984; *Mäkelä et al*,1992). Yokohama et al (1995) was the first to demonstrate using immunolocalisation studies that cells in the interface membranes of loose cemented and cementless prostheses produced MMP-1, MMP-2 and MMP-9. Amongst these MMPs, MMP-9 was strongly positive in most multinucleated giant cells and macrophages. Immunohistochemical examination revealed that the giant cells were strongly positive for MMP-9. There was also an increase of MMP-9 mRNA expression in the cells using *in* situ hybridisation technique. Another study using zymographic and densitometric analysis revealed elevated production of MMP-2 and MMP-9 in tissue extracts from the interface membrane of aseptically loosened implants when compared with non inflamed knee synovial tissue used as controls (Takagi *et al*, 1994). Takei *et al* (1999) demonstrated that the MMP-TIMP profile in the pseudosynovial fluid in aseptic loosening was different from other diseases such osteoarthritis and rheumatoid arthritis. Aseptic loosening is associated with large masses of pseudosynovial membrane (PSM) and this tissue forms an important source of the synovial fluid pool of MMPs. They also noted that aseptic loosening was characterised by the presence of an excess of TIMPs. Takei *et al* (2000) also showed that the mRNA expression for MMP-1, MMP-3, MMP-9, MMP10, MMP-12 and MMP-13 were significantly higher in the interface membrane of

aseptically loosened prostheses compared with synovium taken from osteoarthritis.

In our study, we were able to demonstrate positive staining for MMP-9 in the femora that underwent implantation of ceramic particles. There was also a statistically significant difference between the staining of MMP-9 of femora that had implantation of ceramic particles and sham surgery. The staining was also performed in femora that were retrieved at 10 weeks. Our aim was to quantify the difference in MMP-9 staining between the two different periods and this has not previously been examined. To our surprise, we were not able to demonstrate an increase in the staining of MMP-9 at 10 weeks. Despite this, the p value was approaching the significance level and one possible reason for the decreased staining of MMP-9 is the production of TIMP. TIMP inhibit the activities of MMP. The activity of MMPs is regulated by the balance between MMPs and their inhibitors. This balance has a key role in normal connective-tissue remodelling, and therefore, MMP release, activation and inhibition are usually tightly regulated. The relative balance between MMP and TIMP must ultimately determine the fate of extracellular matrix. It must be acknowledged that MMPs may be in fact beneficial for tissue-healing in view of their role in tissue remodelling. Hence, an imbalance with increased MMP activity may lead to extracellular matrix degradation, but ultimate remodelling is a complex process. MMPs in addition to remodelling of tissue within the periprosthetic connective tissue bed have also been shown in previous studies to be actively involved in bone resorption (Okada *et al*, 1995). The presence of high concentration of MMPs

in the macrophages found in the PSM suggest MMPs may be directly involved in periprosthetic bone resorption (Takagi *et al*, 1994; Yokohama *et al*, 1995; Takei *et al*, 2000).

In our study, we were able to demonstrate an increase in the number of macrophages and osteoclasts in femora that underwent implantation of ceramic particles. This provides evidence that the implantation of ceramic particles induced an inflammatory response but this was not associated with osteoclastic bone resorption in our model. We found no difference in the number of macrophage and osteoclasts in femora implanted with ceramic particles at weeks 4 and 10.

MMPs may represent one final common pathway in bone resorption and aseptic loosening could then potentially be prevented or treated by inhibiting MMPs. Several pharmaceutical agents have been tested in numerous studies to prevent osteolysis. Bisphosphonates have been shown to inhibit bone resorption by PMMA activated macrophages in a radioactive murine calvaria model (Horrowitz *et al*, 1996). Shanbhag et al (1997) also showed that bisphosphonates could prevent bone loss but not the foreign body reaction associated with aseptic loosening in the canine *in vivo* model. Pentoxifylline is an inhibitor of TNF-α and was shown to be as effective as alendronate (bisphosphonate) in preventing debris induced osteolysis in an *in vivo* murine model (Schwarz *et al*, 2000). Tetracyclines have been shown to inhibit MMPs with doxycycline being the most potent inhibitor (Golub *et al*, 1991; Greenwald *et al*, 1987). Tetracyclines are

capable of inhibiting the action of MMPs by a mechanism totally independent of their antibiotic effect. They inhibit MMPs by binding to the enzymes metal dependent site i.e. zinc and cause inactivation. In our department, Ong & Taylor (2003) were able to demonstrate that doxycycline inhibited osteolysis in an *in vitro* model. Our aim was to progress to an *in vivo* model to assess the likely benefits of doxycycline and other pharmaceutical agents. These pharmaceutical agents can then be used in a human trial to assess their clinical benefits.

CHAPTER 6

GENERAL DISCUSSION

The majority of hip joint disease is caused by osteoarthritis. Total joint replacements have been used to alleviate pain and to improve the quality of life for those affected. The total hip replacement is now one of the most important surgical procedures in the world with approximately over 500,000 being performed each year and 60,000 carried out annually in the United Kingdom (Tennant *et al.*, 2000). The aim of the total hip replacement is to mimic as closely as possible the original fully functional hip joint. In order to achieve this, a significant amount of money is spent each year in research on the development of materials, design and testing of hip prostheses. The materials used in hip prostheses must be hard wearing, be able to withstand the stresses exerted at the hip joint and, importantly, be as bioinert as possible.

The most common type of hip prosthesis implanted today consists of a metal femoral head articulating against an UHMWPE socket. The long term survival of this prosthesis is affected by the wear debris generated which induces an inflammatory response, that ultimately leads to osteolysis and implant failure. It is recognised that the biological response to wear debris is one of the main mechanisms of aseptic loosening of metal-UHMWPE THRs. Studies of pseudomembranes from revised prostheses have shown that polyethylene particles are the most frequent component of this debris (Huk *et* al., 1994; Shanbhag *et al.*, 1994). Wear particles are found in high concentrations in the periprosthetic tissues surrounding the implant and are associated with an intense granulomatous reaction. This is characterised by the presence of high numbers of macrophages, which secrete high levels of pro-inflammatory cytokines and other mediators of

bone resorption such as matrix metalloproteinases (Goodman *et al*, 1992; Glant *et al*, 1993; Maloney *et al*, 1995; Takagi *et al*, 1998).

In order to try to reduce the granulomatous response in periprosthetic tissues and subsequently reduce osteolysis and aseptic loosening, alternate bearing materials have been introduced in an attempt to minimise wear associated with artificial hip joints. Materials such as ceramic on ceramic have the theoretical advantage of low friction and therefore a very low generation of wear debris. Ceramic alumina possesses excellent wear characteristics making it ideal for use in a low friction environment such as a total hip replacement. It has been shown that a ceramic femoral head articulating with a ceramic socket produces up to 4000 times less wear volume than a metal-UHMWPE prosthesis (Dorlot *et al.*, 1989). The initial aim of the use of ceramic prostheses was to reduce the wear of the implant components. Another factor in the introduction of ceramic THRs was an attempt to improve the long term results in younger and /or active people and to provide them with a safe, low wearing durable material with an osteolysis free lifespan.

Histological studies of tissues from around metal-UHMWPE prostheses have demonstrated a chronic granulomatous reaction associated with UHMWPE debris local to the implant with the presence of numerous particle laden macrophages and giant cells (Revell *et al.*, 1994). Similar studies of tissues from around metal on metal THRs have also shown a granulomatous reaction, although not as intense as that seen around metal-UHMWPE prostheses with tissue necrosis (Doorn *et al.*, 1998). In this study, we opted to use particles other than metal because of the

associated necrosis and pinocytosis with metal particles and the lack of bone resorption via the production of cytokines and enzymes activated by macrophages because of cell death (Ingham and Fisher, 2000).

With regards to ceramic wear debris in periprosthetic tissue and its *in vivo* effects, data is limited. Several studies have investigated the tissues from around cemented and non-cemented alumina ceramic THRs. These studies have produced conflicting opinions as to the *in vivo* effects of alumina ceramic particles. Kummer *et al.* (1990) and Hensgge *et al.* (1994) suggested that alumina ceramic wear debris does induce an inflammatory response *in vivo*. Huo *et al.* (1995) observed necrosis in tissues around cemented ceramic on ceramic THRs and attributed this to particles of cement. Huo *et al.* (1995) also found no evidence of osteolysis after a mean of 9 years for uncemented ceramic on ceramic prostheses. Shih *et al.* (1994) reported localised femoral osteolysis in 6% of cases of cementless ceramic THRs performed during an average 9 year period. Yoon *et al.* (1998) suggested of 103 uncemented ceramic prostheses, 23 femoral components and 49 acetabular components had radiological signs of osteolysis at a mean implantation time of 92 months.

In this study, our aim was to use clinically relevant ceramic wear particles implanted into the medullary canal of a long bone that would cause osteolysis and this could be accurately quantified by the release of radiolabelled calcium 45. The development of a simple *in vivo* model of osteolysis would allow the testing of potential pharmaceutical agents for aseptic loosening. Reynolds and Dingle

(1970) described an *in vitro* model to investigate bone resorption using radiolabelled calcium 45 murine calvaria. They found this model to be sensitive in the study of hormone induced bone resorption. Several investigators have used this model for bone resorption (Horrowitz & Gonzales, 1997; Horrowitz *et al.*, 1996; Yokohama *et al.*, 1995; Warashina *et al*, 2003; Ong and Taylor, 2003).

It is to be presumed that any particulate debris in tissues local to the artificial hip implant will cause a macrophage infiltrate into the local tissues. With the generation of particulate wear debris from the bearing surfaces of the implant, the macrophage levels will rise. Macrophage presence is associated with the presence of foreign body and ceramic particles should not be classed any differently to any other wear particle in their ability to induce an inflammatory response. It is possible that the size of ceramic wear debris explain a reduced inflammatory response in comparison to particles of UHMWPE. When a macrophage cannot phagocytose or degrade a foreign body, it attempts to recruit more macrophages to the area via the release of cytokines. In the presence of a large amount of wear debris, more cytokines will be released by the macrophages which will cause an osteolytic response local to the implant and instigate bone resorption (Amstutz *et al.*, 1992).

We were initially reluctant to use ceramic particles. It is generally believed that ceramic is a bioinert material, but it has been shown that alumina wear debris has the potential to induce an inflammatory response *in vivo* (Klummer *et* al., 1990; Henssge *et* al., 1994). We initially intended to use polyethylene particles, but

because of difficulties in collection and suspension polyethylene particles were impractical. Therefore, we opted to use ceramic with a clinically relevant size. It has been shown that sub-micrometer sized alumina ceramic particles are capable of stimulating macrophages to produce osteolytic cytokines *in vitro* (Hatton *et al*, 2003). Other histological studies of tissues from around ceramic on ceramic THRs have revealed ceramic particles in the 0.1 - 8μm size range (Yoon *et al.*, 1998; Bohler *et al.*, 2000; Hatton *et al.*, 2002).

It was necessary to remove endotoxin from the particles used, as endotoxin has been shown to stimulate proinflammatory cytokines by macrophages (Krause *et al.*, 1996; Estrada *et al.*, 1998). In our study, the particles were subjected to dry heat treatment at 180°C, which has been shown to effectively destroy surface endotoxin (Tsuji *et al.*, 1978). The particles were then tested using the limulus amebocyte lysate assay (Associates of Cape Cod International). Endotoxin levels were just above the detection limit of the test, which was approximately 0.001 EU per ml. The endotoxin levels present in the debris used were insignificant in their ability to stimulate a cellular response (Ragab *et al*, 1999).

Despite our attempt to develop a small animal model of osteolysis, there are limitations to this model, most obviously the absence of a prosthetic implant. This prohibits the study of mechanical and biomechanical factors that are known to be important in osteolysis. The administration of ceramic particles into a non-weight bearing area of the femur might shield it from the effects of mechanical loading and fluid pressure. Schmalzried *et al* (1992) have shown that mechanical loading

of a total joint prosthesis tends to force synovial fluid into gaps in the interface between the implant and surrounding bone and enhance the passage of particulate wear debris around the bone – implant interface. Another limitation of why we were unable to develop this small animal model of osteolysis is that we depended on the implantation of a bolus of ceramic particles. These circumstances are not representative of the human situation, where wear debris are produced continuously and a chronic response is induced in periprosthetic tissues. Other possible reasons for minimal osteolysis in our model could be the possibility that hyaluronic acid inhibited wear debris induced osteolysis and that we did not wait long enough after the implantation of the ceramic particles to detect osteolysis.

Despite all the above limitations, our aim was to develop a small animal model of wear debris induced osteolysis that could be used to screen potential pharmaceutical agents such as bisphosphonates or matrix metalloproteinases inhibitors to prevent bone loss associated with wear debris. The important potential differences with our model were the decreased cost and simplicity of the model even though our model failed to work.

The second part of the study was to investigate whether urinary NTx and DPD could be used as bone markers in our model. These markers have been used in the past in other models. The aim of this part of the study was to demonstrate that these urinary bone markers could possibly be used to monitor resorption activity and especially during pharmaceutical treatment. Unfortunately, the urinary levels of NTx and DPD were not significantly different in animals that were implanted

with ceramic particles compared to animals that underwent sham surgery. This could be explained by the bioinertness of ceramic particles or the lack of sensitivity in our model. We were also unable to correlate the urinary NTx and DPD with the scintillation counts of the femora that underwent implantation of ceramic particles.

With regards to the staining of bone for matrix metalloproteinase-9, we were able to demonstrate a difference in the staining between animals that underwent implantation of ceramic particles and whose who underwent sham surgery at 4 and 10 weeks. We were unable to detect a difference in the quantity of MMP-9 staining between 4 and 10 weeks in femora that underwent implantation of ceramic particles. However, the implantation of ceramic particles into femora increased the number of macrophages and osteoclasts compared to control femora. This provides some evidence that the ceramic particles induced an inflammatory response even though this was not associated with bone resorption. We are not aware of any other study that has quantified the amount of MMP over time. We can only speculate that the production of TIMP occurred over time and might have decreased the amount of MMP-9 at 10 weeks.

Currently, there are no pharmaceutical agents licensed for the use in wear debris induced osteolysis. Alendronate, a third generation bisphosphonate has been shown to inhibit osteolysis in an *in vivo* model (Schwarz *et al.*, 2000). Pentoxifylline, an agent that inhibits the responses of TNF-α to wear debris has also been shown to decrease wear debris induced osteolysis in an *in vivo* animal

model (Schwarz *et al*, 2000). More recently, doxycycline that is used as an antibiotic has been shown to inhibit osteolysis in an *in vitro* model (Ong & Taylor, 2003). Both doxycycline and simvastatin have the abilities to inhibit matrix metalloproteinases. Both these agents have proven safety records for human usage in other medical conditions.

Further investigation into the histological appearances of tissues from around ceramic on ceramic THRs needs to be performed to attempt to establish the effect of ceramic wear debris *in vivo*. Techniques such as immunocytochemistry and immunohistochemistry for the detection of cytokines and enzymes such as matrix metalloproteinases should be performed to improve the understanding of the biological reactivity to ceramic particles.

Further studies are required to investigate the efficacy of these agents *in vivo* in the inhibition of wear debris induced osteolysis to allow guidance on the optimal choice of agents in eventual human trials. Small animal models that are cost effective and that permit rapid analysis of biologic responses have been very important for the development of pharmaceutical agents in other areas of biomedical research. However, we recognise the need for eventual studies in humans to confirm effectiveness of these agents.

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APPENDIX 1

Grant Awards

1. **Zimmer Ltd**

   Awarded a sum of £5000.

2. **Foxtrot**

   Awarded a sum of £2500

Presentations

1. Ibrahim T, Ong SM, Taylor GJS.

   Quantitative analysis of osteolysis in a small animal model of aseptic loosening.

   The British Orthopaedic Research Society, Manchester, England, 2004.

2. Ibrahim T, Ong SM, Taylor GJS.

   The utilisation of NTx and DPD as bone markers for osteolysis in a small animal model of aseptic loosening.

   The British Orthopaedic Research Society, Manchester, England, 2004.

3. Ibrahim T, Ong SM, Taylor GJS.

   The development of a small animal model of wear-debris induced osteolysis.

   The European Orthopaedic Research Society, Bologna, Italy, 2006.

4. Ibrahim T, Ong SM, Taylor GJS.

The utilisation of urinary bone markers in a small animal of wear-debris induced osteolysis.

The European Orthopaedic Research Society, Bologna, Italy, 2006.

5. Ibrahim T, Ong SM, Taylor GJS.

   Matrix metalloproteinases-9 in a small animal model of wear-debris induced osteolysis.

   The European Orthopaedic Research Society, Bologna, Italy, 2006.

APPENDIX 2

Name and addresses of major supplies

Amersham Biosciences UK Ltd.,

Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA.

Associates of Cape Cod International,

Deacon Park, Moorgate Road, Knowsley, Liverpool, Merseyside L33 7XL.

Baxter Healthcare Ltd.,

Wellingford Road, Compton, Newbury, Berkshire RG20 7QW.

Biomet Merck Ltd.,

Waterton Industrial Estates, Bridgend, South Wales CF31 3XA.

Chemicon Europe,

Eagle Close, Chandlers Road, Southampton, Hampshire SO53 4NF.

Dako Ltd.,

Denmark House, Angel Drove, Ely, Cambridgeshire CB7 4ET.

Fisher Scientific UK,

Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG.

Quidel Diagnostics,

40 Church Road, Wheatley, Oxford OX33 1NB.

Sigma-Aldrich Co Ltd.,

Fancy Road, Poole, Dorset BH17 7NH.

VWR International Ltd.,

Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN.

Whatman International Ltd.,

St Leonard's Road, Maidstone, Kent ME16 0LS.