REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree MD Year 2006 Name of Author ADELINTOU J. B

An Investigation of the Osteoinductive Properties of Colloss

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SUBMITTED FOR THE DEGREE OF DOCTOR OF MEDICINE

(M.D.) at THE UNIVERSITY OF LONDON

2005

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ABSTRACT

Colloss is a sterile extract of bovine bone matrix which consists predominantly of collagen type 1, but also contains several growth factors associated with bone matrix. The ability of Colloss to stimulate bone formation adjacent to titanium implants *in vivo* and its effect on the proliferation and differentiation of multipotential human bone marrow stromal stem cells (BMSSCs) *in vitro* and on titanium and hydroxyapatite (HA) was investigated.

When BMSSCs were cultured with a solution of Colloss in vitro for 21 days, evidence of osteogenic differentiation was signified by the expression of Alkaline Phosphatase (ALP), collagen type I, calcium deposition and changes in cell morphology.

When BMSSCs were cultured on titanium pre-treated with Colloss, there was increased expression of the osteoblastic markers ALP at day 7 and osteopontin on days 14 and 21 compared to BMSSCs cultured on untreated titanium. BMSSCs cultured on porous hydroxyapatite pre-treated with Colloss showed no difference in the expression of the osteogenic markers ALP and osteopontin compared to BMSCCs cultured on untreated porous hydroxyapatite. However there was an increase in collagen type I synthesis, which was 3 times that of control cultures of BMSSCs on untreated HA at 21 days.

When Colloss was used to fill 1 and 2 mm defects adjacent to shot blasted titanium implants placed in tibia of sheep, there was no difference in the area of new bone formation or the degree of mineralisation of bone between the control and Colloss containing defects.

These findings show that Colloss can induce the osteogenic differentiation of bone marrow stromal stem cells cultured in monolayer and on smooth polished titanium. Results of BMSSCs cultured on hydroxyapatite were inconclusive, although evidence of collagen type I synthesis was suggestive of matrix deposition. Colloss did not enhance bone formation within defects adjacent to titanium implants *in vivo*.

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1.Introduction

The successful treatment of many orthopaedic conditions is dependent on the biological process of bone formation. Occasionally the biological bone healing response is insufficient and surgical intervention is required to promote formation of osseous tissue. For example, surgical intervention for the treatment of fracture non-unions often involves the placement of graft material at the fracture site. In other cases, the enhancement of bone formation is desirable to improve the stability of large load bearing orthopaedic implants. For instance, around failed hip replacements bone loss is common adjacent to the femoral stem resulting in periprosthetic defects. When this occurs, the technique of impaction bone grafting can be used to stimulate bone formation within these defects to enhance implant osseointegration and stability.[1]

Three fundamental factors which enhance bone formation are osteoinduction, osteoconduction and osteogenesis. Osteoinduction is the process by which there is stimulation of the differentiation of osteoprogenitor cells into osteoblasts and subsequent bone formation. Osteoconduction describes the ability of a substance to support the migration and proliferation of osteoprogenitors and osteogenicity signals the presence of bone forming cells.

Autologous bone graft has these three properties (osteoconductivity, osteoinductivity, osteogenicity) and is considered the gold standard graft material. It has an osteoconductive lattice structure comprising hydroxyapatite and collagen, and within its matrix are osteoinductive growth factors which stimulate osteogenesis.[2] It is also osteogenic, containing cellular components comprising osteoblasts and osteoblast precursors which are required for osteosynthesis.

Unfortunately the use of autograft is associated with morbidity relating to the harvest site and increased surgical time. Furthermore its supply is limited and in certain

procedures the amount of graft available may be insufficient. Younger et al [3] reviewed the outcome of 239 patients undergoing bone grafting during orthopaedic procedures and found and overall complication rate of 8.6%. Complications included infection (2.5%), haematoma (3.3%), persistent pain (2.5%) and sensory loss (1.2%).

Allogenic bone graft, demineralised bone matrix and various synthetic bone graft substitutes have been used as alternatives to autologous bone graft, but in many cases have been less effective in promoting bony union.[4,5,6] Whilst these alternatives may have osteoconductive and / or osteoinductive properties, when used without additional growth factors, they are less osteoinductive than autologous bone graft. Furthermore, they do not contain osteogenic cells required for bone formation and instead rely on migration of cells from the host at the site of implantation. In addition, with the use of allogenic graft there is risk of disease transmission.[7] For these reasons, there has been intense research to develop graft materials with improved efficacy.

In the clinical context, methods of generating osseous tissue would be valuable in any situation where bone formation is desirable. This would include the healing of fractures and fracture non-unions, the formation of bone within large long bone defects secondary to injury or disease and the fusion of spinal vertebrae

Tissue engineering is a discipline in which cells, growth factors and biomaterials are utilised to repair or replace human tissue. The term "tissue engineering" was officially coined at a National Science Foundation workshop in 1988 to mean "the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function".[8]

The generation of viable tissues is based on strategies which utilise cells and growth factors in combination or independently and a suitable carrier. Crucial to the success of

any tissue engineering technique is the development of a suitable delivery system for the cells and growth factors utilised for the generation of tissue. For bone tissue engineering, carriers often take the form of biodegradable scaffolds which are based on collagen or polyglycolic acid.[9,10,11] Ideally a carrier should have a capacity to hold and retain a large number of cells and or growth factors and have the structural integrity to withstand forces generated at the site of implantation. It should also be osteoconductive, promoting ingrowth of tissue at the host – carrier interface. The carrier should gradually be resorbed and replaced by host tissue. In general, tissue engineering strategies for tissue repair and regeneration involve the generation of tissue *in vitro* for implantation, or the implantation of a material which induces the formation of the desired tissue *in vivo*.

1.1 Colloss (collagen lyophilisate)

Bone consists of cells and extracellular matrix. The extracellular matrix is 35% organic and 65% inorganic. The organic part is 90% collagen. The other 10% comprising osteocalcin, osteonectin, glycosaminoglycans and lipids. The inorganic part consists of calcium complexed with phosphate as hydroxyapatite. Bone matrix contains many of the growth factors involved in the cascade of bone formation. Bone matrix can be demineralised by treatment with hydrochloric acid to form demineralised bone matrix (DBM). The ability of DBM to induce new bone formation has been demonstrated in several animal studies.[12,13,14] In humans it is often used as a graft for maxillo-facial reconstruction procedures.[15,16]

Colloss (collagen lyophilisate, Oberstenfeld, Germany) is a sterile extract of bovine bone matrix. It is white and has a cotton wool consistency. There are several steps involved in the production process. First bovine bone undergoes a degermination (removal of cells) procedure involving treatment with hydrochloric acid and acetone. It

is then milled and under aseptic conditions undergoes decalcification and delipidation. The final product is obtained by an extraction process and then it is freeze dried. Colloss consists predominantly of collagen type 1, but also contains many of the osteogenic growth factors associated with bone matrix in a concentrated form. These factors include bone morphogenic proteins which can induce bone formation. Collagen type I is osteoconductive[10] and has binding sites for cellular attachment.[17] It can also promote calcium deposition by providing binding sites for mineral deposition and by binding non-collagenous proteins such as osteonectin and osteocalcin which promote calcium crystal deposition.[18] Collagen also binds osteogenic growth factors. There are anecdotal reports of the use of Colloss to promote bone formation when used in clinical practice for the treatment of phalangeal cysts and for reconstructive maxillofacial procedures.

The ability of Colloss to enhance bone formation adjacent to implants was demonstrated in a study by Schlegal AK et al 2003. Ankylos dental implants coated with Colloss were placed in the frontal bones of pigs. In a further 2 groups of animals, implants were coated with platelet rich plasma or placed in an implant bed in which peri - implant bone had been condensed. In the control group there was no preparation of the implant or implant bed. Outcome measures were bone formation and bone density at the bone implant interface. At 2 weeks interface bone was greater in the test groups than the control group and the Colloss group was 60% compared to 30% in the control group. Similarly, at 4 weeks the Colloss group showed more implant interface bone than the control group, but by 6 weeks implant interface bone was similar between all groups. Bone density was greater in the Colloss group than control at both 2 weeks and 8 weeks.[19]

In a further study by Wiltfang et al 2004, Colloss was implanted into critical sized defects in the skull bones of pigs.[20] In comparison with an empty control defect, the

degree of reossification was 52.6% at 2 weeks compared to 38% in defects filled with autologous bone, 43.1% in Bio Oss (Bone substitute) filled defects and 32% in Cerasorb (bone substitute) filled defects. When BMP 2, which is expressed during early osteogenesis, was measured in the Colloss filled defects at 2 weeks, it was 7 times greater than in the surrounding bone. In a similar experimental model, Schlegel et al found Colloss to be comparable to autologous bone for ossification of bone defects.[21] The ability of Colloss to enhance bone formation in these experiments, without the addition of osteoprogenitors, suggests that it has a greater osteoinductive effect on local host cells than bone graft or bone substitute materials.

1.2. Bone marrow stromal stem cells (BMSSCs)

Bone marrow stromal stem cells are primitive cells present within bone marrow where they account for approximately 1 per 100,000 of the nucleated cells.[22] They are multipotential, hence have the ability to differentiate along several lineage pathways to form a variety of tissue types including tendon, fat, muscle, cartilage and bone.[23,24] These properties raise the possibility of utilising such cells to repair skeletal tissues. As a result there has been intense interest in bone marrow stromal stem cells with investigation of their multipotential characteristics and behaviour in various cell culture environments.[24-26]

1.3. Titanium and the Osseointegration of Orthopaedic Prostheses

For several years titanium has been used for the manufacture of orthopaedic implants.[27-30] Pure titanium can be combined with other metals, such as vanadium and aluminium, to form titanium alloy. The alloy Ti-6%AL-4%V was initially developed for the aerospace industry, but now is used for the manufacture of orthopaedic

implants. The alloy is much stronger, with an ultimate tensile strength of 1170 Mpa compared to 220 Mpa for pure titanium.

Titanium and its alloys have physical and biological properties which are desirable. Its physical properties are such that it is easy to machine into components, thus reducing the time and expense of implant manufacture. It has a modulus more similar to bone than other currently used implant alloys which, minimises the stress shielding effects of the implant and reduces the associated bone resorption.[31] This may reduce the risk of implant loosening and periprosthetic fractures. When exposed to oxygen, titanium reacts to form an oxide layer which provides protection from corrosion.[32]

Unfortunately titanium alloys also display unfavourable characteristics which limit their application in orthopaedic practice. It has poor wear properties and hence is not routinely used as a bearing surface unless it has undergone surface modification.[33, 34,35] It is sensitive to notching, which can significantly reduce its fatigue strength, and there is also concern about the toxicity of titanium alloys used for prosthesis manufacture.[36,37]

Loosening of the femoral component of total hip replacements limits the longevity of prostheses. Studies have shown radiographic loosening rates in uncemented prostheses of 2-27% at follow up of 5 to 13 years.[38,39,40,41,42] The aetiology of loosening has been investigated in several studies. It has been demonstrated that a key factor in the loosening process involves the generation of polyethylene wear particles at the interface between the acetabulum and femoral head prosthetic components.[43,44] These particles migrate down the cement – bone interface or implant – bone interface (uncemented femoral stems), where they are engulfed by macrophages. Particles less than 10μm can be phagocytosed by macrophages which respond to these particles by releasing a variety of inflammatory mediators, including interleukin 1β, IL6, TNFα and PGE2, which stimulate bone resorption thereby accelerating the loosening

process.[45,43,46,47,48,49] Eventually a fibrous membrane forms between the implant and bone. Therefore it is reasonable to propose that by creating a seal between bone and the implant, the migration of polyethylene wear particles can be prevented and the loosening process can be subdued.

In the field of maxillofacial surgery, considerable progress has been made in achieving osseointegration of titanium orthodontic implants.[50,51] There has been less success in achieving the osseointegration of large functional orthopaedic prostheses, where the mechanical environment is more demanding. In an attempt to improve osseointegration, various modifications have been made to titanium alloys with mixed results. These include altering its chemical structure[52], making topographical changes or coating the implant surface with materials to improve bony ingrowth.

Variations in the surface roughness of titanium have been shown to influence the behavior of osteoblastic cells. [53,54,55,56] Shot blasting or sandblasting the surface of implants to create undulations in their surfaces can improve the osseointegration of titanium implants. [57,58]

Coating titanium with certain materials has also been effective in improving bone ingrowth. These include hydroxyapatite, sintered beads and wire mesh. Hydroxyapatite is a bioactive material which has been shown to have osteoconductive properties. When used to coat titanium implants, it promotes bone ingrowth at the host implant interface. [59,60,61] Unfortunately there are problems associated with the use of HA as an implant coating. Long term studies have shown that the function of HA is limited by compromise of bonding between HA and the implant, leading to delamination and resorption of the HA coating.[62-64]

1.4. Aim

The aim of this study is to investigate the osteogenic potential of Colloss on bone formation and to establish its effect on the behaviour of bone marrow stromal stem cells cultured in vitro. The effects of Colloss on the growth and differentiation of bone marrow stromal stem cells cultured in vitro was investigated in monolayer and on titanium and hydroxyapatite biomaterials. An *in vivo* study was then performed to assess the effect of Colloss on bone formation within defects adjacent to titanium alloy implants placed in sheep tibia.

1.5. Hypotheses

1. Colloss will stimulate the osteogenic differentiation of bone marrow stromal stem cells cultured in monolayer and on titanium and hydroxyapatite biomaterials.
2. Colloss can stimulate bone formation within defects adjacent to titanium implants enhancing their osseointegration.

2. LITERATURE REVIEW

2.1. History

The use of cells for the purpose of healing bone was documented more than 300 years ago, in 1668 by Job Van Meek'ren. Two hundred years later in 1869, Gougon, described the osteogenic potential of bone marrow.[65] In more recent times bone marrow has been used successfully as a graft material to heal tibia, scaphoid and humeral fracture non-unions.[66-68, 69, 70] In 1991 Connolly described the use of bone marrow as an alternative to bone graft for the treatment of non-unions. In Connolly's study the healing of 18 of 20 ununited tibia fractures was achieved by injecting bone marrow aspirates directly into the fracture site. [71]

Direct evidence of the presence within bone marrow of cells with osteogenic potential was provided by Friedenstein in 1966 who showed that in a diffusion chamber single cell marrow suspensions could form mineralised bone.[72] Further evidence to support the existence of osteoprogenitor cells within bone marrow was provided in experiments by Friedenstein and Ashton,[73-75] who demonstrated that guinea pig and rabbit marrow fibroblast cultures formed bone when transplanted into diffusion chambers. It was later proposed that osteoprogenitors within the bone marrow were derived from stromal stem cells which have the potential to differentiate into several cell types to form mesenchymal tissues.[23]

More recently, studies have demonstrated that within bone marrow there are cells capable of differentiating to form adipocytes,[76] chondrocytes[77] and bone.[78] Athough it had been demonstrated that there were cells within bone marrow which could differentiate to form several cell types, it was unclear as to whether such tissues originated from a true multipotential stem cell or were the product of several different progenitors. In 1999 Pittenger et al [23] performed a study which gave strong support to

the presence of a true mesenchymal stem cell. Bone marrow stromal stem cells (BMSSCs) were isolated from human donors and cultured in monolayer. Individual cells were identified in culture and grown to form colonies. Colonies, which had therefore originated from one single cell were expanded in culture, then using media supplements were stimulated to differentiate along lineage pathways to form adipocytes, chondrocytes and osteocytes.

Stem cells by definition are undifferentiated, self replicating and multipotential. The presence of bone marrow stem cells with such properties means there is great potential for the regeneration of skeletal tissue and thus the treatment of patients suffering from disease or injury.

2.2. Bone marrow stromal stem cells

Mesenchymal stem cells can be found in muscle,[79,80] periosteum[81,82] and adipose tissue [83,84,31,85] but are most commonly obtained from bone marrow (bone marrow stromal stem cells) because of the ease of harvest. Bone marrow stromal stem cells (BMSSCs') are the precursors of osteoprogenitors. After skeletal maturity the number of osteoprogenitors within bone marrow decreases. This was demonstrated by Nishida et al 1999. The presence of osteoprogenitors was determined by quantifying Alkaline Phosphatase expression. It was found that although the ability of bone marrow stromal stem cells to form osteoblasts was maintained with advancing age, there was a marked drop in number of these cells after the first decade of life, followed by a more gradual decline after age twenty. [86] D'ippolito et al 1999 came to a similar conclusion from the examination of vertebral bone marrow.[87] It is possible that these observations are a contributory factor in the age related reduction in the capacity of fractures to heal and may also be a mechanism for senile osteoporosis.

Following harvest, BMSSCs can be expanded in culture and passaged up to 38 times without loss of phenotype or ability for multipotential differentiation.[88] BMSSCs in culture have been differentiated in vitro to form adipocytes, chondrocytes, fibroblasts and osteoblasts by the addition of supplements to growth medium. The osteogenic differentiation of bone marrow stromal cells has been achieved using dexamethasone, β glycerol phosphate and ascorbate.[23,78]

2.3. Clinical application of stem cells

Following expansion of cells in culture, there are several options with regards to how they are utilised to generate skeletal tissue. They can be delivered to a skeletal site as a cell suspension or combined with a carrier material. Growth factors can be used to stimulate the differentiation of mesenchymal stem cells along desired lineage pathways prior to implantation, or can be applied to cells at the time of implantation. However many growth factors are short acting and when delivered *in vivo* their biological activity may be of insufficient duration to achieve effective healing. To overcome this problem gene therapy has been utilised in studies to produce bone marrow stem cells which can express specific growth factors.[89,90] Mesenchymal stem cells have been used to repair tendons, and heal articular cartilage and long bone defects.[91,9,92]

2.3.1. Cartilage defects

Wakitani et al 1994 used mesenchymal stem cells harvested from the bone marrow and periosteum of rabbits to heal articular cartilage defects.[91] Cells were expanded in culture then dispersed in a collagen gel prior to implantation into large cartilage defects on the weight bearing surface of rabbit femoral condyles. In control animals defects were left empty. At 2 weeks implanted osteoprogenitor cells had differentiated into

chondrocytes and at 24 weeks the tissue in the cell filled defects was stiffer than that of the empty defects but less stiff than the normal cartilage.

2.3.2. Tendon

Young et al 1998 used mesenchymal stem cells to heal defects in rabbit Achilles tendons.[9] Mesenchymal stem cells were aspirated from bone marrow, expanded in culture, and then suspended in a collagen gel which was loaded onto a suture prior to implantation within the tendon defect. In the control group tendon gaps were filled only with suture material. Tendons were evaluated histologically and biomechanically at 4, 8 and 12 weeks. At all three time intervals, the cross sectional area of the treated tendons was significantly greater than the control group. At 4 weeks tendon stiffness in the treated group was 54.2% of normal tendon and twice that of control tendon. These values remained constant to the 12 week end point of the study. On histological examination repair tissue in the experimental group consisted of organised bands of collagen similar to tendon fibroblasts. In contrast, control specimens showed a variable morphology.

In both these studies bone marrow stromal cells differentiated into location specific tissue without the addition of growth factors. This suggests that local environmental signals play a role in determining the differentiation lineage pathway.

2.3.3. Bone formation

Bone formation is a complex process which involves the differentiation of cellular precursors into osteoblasts and osteocytes with the formation of mineralised matrix. During the cascade of osteogenesis, bone marrow stromal stem cells differentiate to form osteoprogenitors, then pre-osteoblasts and finally osteoblasts. During this process there are changes in cellular activity. There is an early phase of cell proliferation which is followed by matrix synthesis by osteoblasts. In the early phase of matrix synthesis, unmineralised osteoid accumulates. Later, calcium accumulates and forms complexes with phosphate resulting in matrix mineralisation. During the process of osteogenesis certain markers of osteoblastic differentiation are expressed. These include alkaline phosphatase, collagen type 1, osteocalcin, osteonectin and osteopontin.[18] These markers can be quantified in vitro, allowing investigators to monitor the osteogenic process.

The utilisation of BMSSCs to stimulate bone formation has been of particular interest because it may provide a means of enhancing new bone formation, providing an alternative to autologous bone graft. Bone marrow stromal stem cells have been used in animal studies to heal segmental long bone defects in animals. Bruder et al, 1998, created 21mm long segmental defects in canine femora.[92] In the experimental group, defects were filled with porous ceramic cylinders which had been loaded with autologous culture expanded mesenchymal stem cells. In the control group, defects were filled with porous ceramic cylinders alone. At sixteen weeks animals were killed and specimens harvested for examination. In the control group, all femora went on to non-union. In contrast all the defects treated with BMSSC loaded ceramics formed a collar of new bone surrounding the implant and had united radiographically by 16 weeks. In other studies BMSSCs have been used successfully to heal segmental defects in the rabbit radius [93] and rat femur.[25]

2.4. Growth Factors involved in the cascade of bone formation

Growth factors are small protein molecules which interact with cell surface receptors to influence cell behaviour. During the process of tissue formation, growth factors influence cell proliferation, differentiation and matrix production (fig.1). Several growth factors which are involved in the cascade of bone formation have been identified and their properties investigated. These include fibroblast growth factor (FGF), insulin like growth factor (IGF), platelet derived growth factor (PDGF) and the transforming growth factor beta (TGFβ) superfamily which includes the bone morphogenic proteins (BMPs).

2.4.1. Transforming growth factor Beta (TGF β)

Transforming growth factor β(TGF β) is believed to play an important role in bone growth and repair. This is supported by the positive immunostaining of TGF β in fracture callus during the healing process.[94] TGF β is a sulphydryl bonded dimeric molecule, of which there are 5 isoforms. It is present in chondrocytes, platelets, osteoblasts and mesenchymal stem cells and is secreted in the form of a propeptide which is enzymatically cleaved to release the active molecule. On secretion, TGF β is sequestered by a binding protein which modulates its interaction with receptors. All forms of TGF β interact with cells via type I and II receptors. TGF β receptors are serine / threonine kinases and on activation, their effects on the target cell nucleus are mediated by intercellular proteins called SMADS. The effects of TGF β on mesenchymal stem cells has been studied in vitro. Cassiede et al investigated the effects of TGF β1 on alkaline phosphatase expression, cell proliferation and osteochondrogenic potential of pre-confluent rat mesenchymal stem cells.[95] In the experimental group MSCs were cultured with TGF β1 5ng/ml for 48 hours. Non-treated mesenchymal stem cells were used as controls. Mesenchymal stem cells pre-

treated with TGF β1 showed increased proliferation rates when compared to the control group. However, Alkaline phosphatase levels were lower in the TGF β1 pre-treated group. Pre treated and control MSCs were then either cultured in osteogenic medium or seeded onto porous calcium phosphate and implanted subcutaneously in synergeic rats. Assay for in vitro nodule formation indicated that TGF β1 inhibited bone formation. However, in vivo it supported the formation of cartilage and bone.

In vivo, the effect of TGF β on the repair of bone has been studied in animal models. Nielsen et al 1994 injected TGF β at a dose of 4 or 40ng around the fracture site of healing rat tibia fractures.[96] The 4ng dose had no effect on bone strength when compared to the placebo, however rat tibia injected with 40ng TGF β had a higher ultimate load and increased amount of bone formation at the fracture site. Lind et al, 1993, [97] examined the effects of TGF β on the healing of tibial defects in rabbits. TGF β was applied at doses of 1 and 10 micrograms per day in 2 groups of rabbits with plated mid tibial osteotomies. TGF β resulted in an increase in bending strength and callus formation, but no effect on bending stiffness, bone mineral content, cortical thickness or Haversian canal diameter. The authors concluded that TGF β enhanced fracture healing in rabbits.

2.4.2. Bone Morphogenic Proteins (BMP)

In 1965 Urist described the osteoinductive properties of demineralised bone matrix. In his initial experiment, bone formation was stimulated by the implantation of demineralised bone matrix into subcutaneous pouches in rats.[98] Later, the proteins responsible for the osteoinductive properties of demineralised bone matrix were identified and called the bone morphogenic proteins (BMPs). They are a group of 15 low molecular weight glycoproteins which belong to the TGF β superfamily. Unlike TGF β, they are secreted in the active form. They have ligands for the type I and II cell

surface receptors. When BMPs interact with type I receptors, type 2 receptors bind with and phosphorylate the type 1 receptor. This interaction and the resulting complex results in phosphorylation of intracellular SMAD proteins, which are involved in intracellular signal transduction.[99]

The bone morphogenic proteins stimulate the differentiation of mesenchymal stem cells along osteochondrogenic pathways to form bone. They play an important role in the induction of bone formation during fracture healing. The identification of the genetic sequence for BMP has allowed its synthesis *in vitro* using recombinant gene technology. [100] There has been intense interest in the bone morphogenic proteins since they were discovered, and many animal studies have been performed to assess their ability to stimulate bone formation and heal skeletal defects in vivo and in vitro. Itoh et al, 1998, investigated the ability of human recombinant BMP 2 to heal segmental long bone defects in dogs.[11] Two centimetre defects were created in bilateral ulnae of 8 dogs. The defect was plated then filled with a poly D, L lactic – co-glycolic acid (PGLA)/gelatin sponge complex (PGS) carrier, either alone or in combination with 40μg, 160μg or 640μg recombinant BMP 2. There were 4 ulnae per treatment group. Twelve weeks postoperatively all defects treated with 160μg and 640μg BMP 2 showed radiographic evidence of union. The group treated with 40μg showed bone formation but no healing, and the control group treated with carrier alone did not demonstrate any evidence of healing. Bone mineral content at 16 weeks was significantly higher in the groups treated with 640μg and 160μg BMP than the defects filled with 40μg BMP 2 or PGS carrier alone. Cook et al 1995 investigated the healing of ulna and tibial defects in monkeys using OP 1 (BMP 7).[101] Ulna defects were filled with either 1000μg of OP 1 combined with carrier, autologous bone graft or carrier alone. Five of the six ulnae treated with OP 1 had healed radiographically at 6 to eight weeks, whereas none of the group that received the carrier alone or autologous bone

graft showed evidence of complete healing. On mechanical testing, ulnae treated with OP 1 had a torsional strength which was 92% that of the contralateral ulnae, whereas ulnae treated with autologous bone graft had insufficient healing and therefore could not be mechanically tested.

2.4.3. BMPs in humans

BMP 2 and BMP 7 have recently gained approval from the FDA (United States, food and drug administration) and are currently licensed for use in humans as an alternative to autologous bone graft in spine surgery and to treat long bone non-unions respectively. Results of early trials using BMP 2 in spinal surgery have been promising. When used as a graft for the lumbar and cervical spine, fusion rates were comparable with those of autologus bone graft.[102]

2.4.4. Insulin like growth factor (IGF)

There are 2 types of insulin like growth factor. These are IGF I and IGF II. IGF I mediates many of the functions of growth hormone and is involved in osteoblast and chondrocyte proliferation, differentiation and matrix formation. IGF II is involved in the growth of foetal tissue and the differentiation of osteoblasts and chondrocytes.

Trippel et al, 1993, demonstrated the stimulatory effect of IGF I on chondrocytes from the distal radial growth plates of calves.[103] Proliferation was measured by 3H thymidine incorporation and matrix production by 35S-sulphate incorporation into glycosaminoglycans. IGF I was shown to increase thymidine incorporation by 2.5 X control cultures and increase 35S sulphate incorporation by 2.6 X control cultures. Scheven et al, 1991, examined the effects of recombinant IGF I and II on human osteoblast cells derived from femoral heads and found both to have a stimulatory effect of osteoblast cell growth.[104] *In vivo*, circulating insulin like growth factor is bound to

IGF binding proteins, of which there are six. They act, not only to transport IGF to target tissues but also have a role in determining the bio availability of IGF. There is also evidence to suggest that IGF Binding proteins may act independently as growth factors to stimulate bone formation.[105]

OSTEOGENESIS

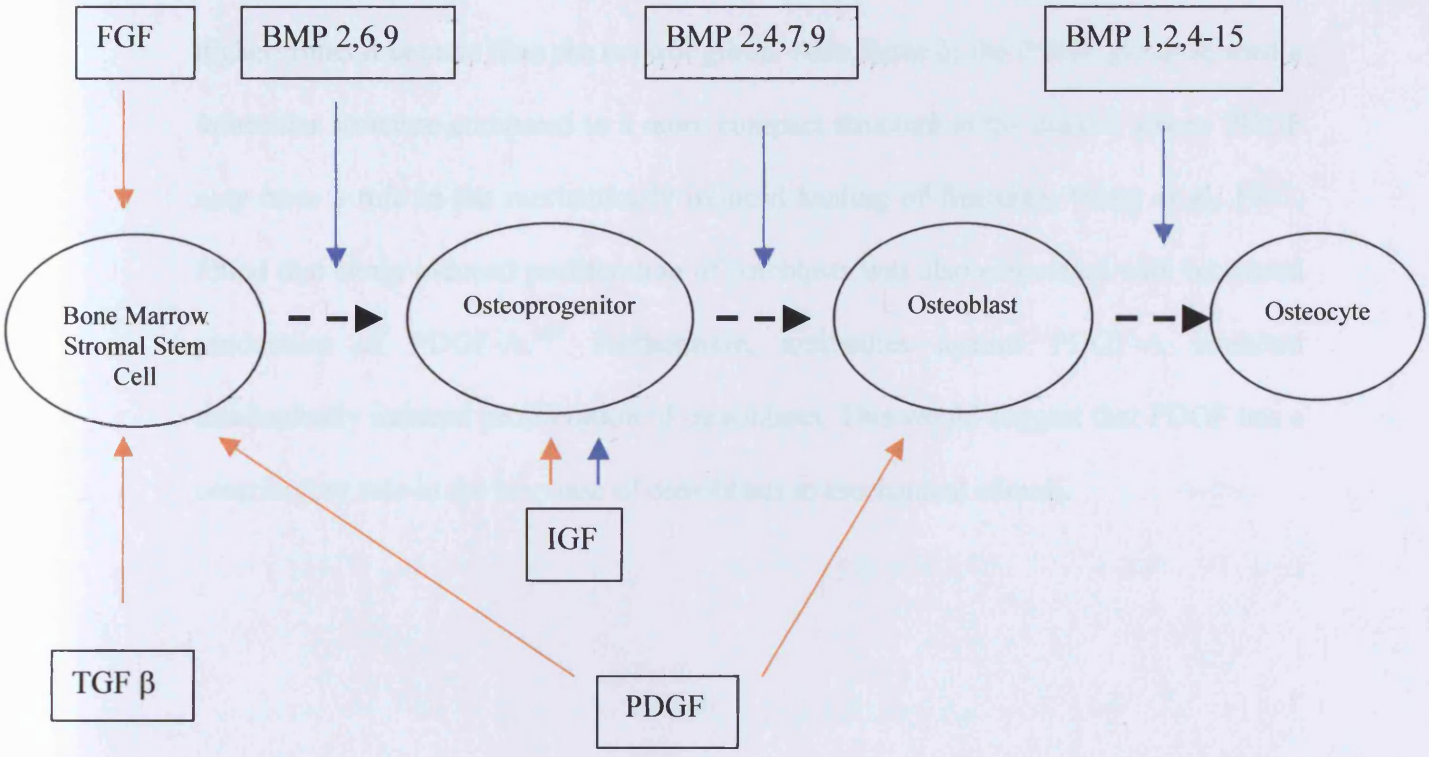


Fig.1. Substances which influence cell proliferation and differentiation during osteogenesis

2.4.5. Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor is a glycoprotein which is released from platelets and mesenchymal cells. It has been shown to stimulate osteoblast proliferation in vitro and bone formation in vivo, and is believed to be involved in fracture healing, although its precise role has still to be clearly defined. Vikjaer et al, 1997,[106] investigated the ability of recombinant PDGF to heal calvarial defects created in 16 rats. Eight defects were filled with a methylcellulose gel combined with recombinant PDGF. In the control group, defects in 8 rats were filled with methylcellulose gel alone. Bone formation was evaluated at 8 weeks. It was found that PDGF stimulated more bone ingrowth with a higher mineral content than the control group. Also, bone in the PDGF group formed a trabecular structure compared to a more compact structure in the control group. PDGF may have a role in the mechanically induced healing of fractures. Wang et al, 1997, found that strain induced proliferation of osteblasts was also associated with increased production of PDGF-A.[107] Furthermore, antibodies against PDGF-A inhibited mechanically induced proliferation of osteoblasts. This would suggest that PDGF has a contributory role in the response of osteoblasts to mechanical stimuli.

2.4.6. Fibroblast growth factor (FGF).

Fibroblast growth factors (FGFs) belong to a family of nine structurally related polypeptides. They interact with the cell surface receptors of a variety of cells, including osteoblasts, chondrocytes, epithelial cells and myocytes. The most common types of FGF found in humans are FGF-1 and FGF-2. There are 4 cell membrane spanning receptors through which FGF interacts to influence cell function. The effects of FGF have been studied in vitro and in vivo. Kato et al 1998 made 3mm segmental defect in the tibia of rabbits, which were then injected with recombinant FGF-2 in varying concentrations.[108] At 5 weeks bone formation within the defects was examined. At concentrations of 100μg and above FGF-2 increased bone volume and mineral content in the defects.

Nakamura T et al 1998 investigated the effects of FGF-2 on fracture healing in dog tibia. Transverse fractures were created in dog tibiae.[109] In the experimental group fractures were injected with 200μg of FGF-2. At week 4 the FGF-2 injected fractures had a greater amount of callus and at week 8 a greater bone mineral content than the control group. At weeks 2 and 4 FGF-2 also resulted in an increase in the number of osteoclasts in the periosteal callus. The authors concluded that FGF-2 promotes both fracture healing and remodelling.

The effect of FGF-2 on fracture healing in primates has also been investigated. Radomski, et al injected a single dose of FGF-2 combined with hyaluronan into 1mm osteotomies in the fibulae of baboons.[110] The callus area was significantly greater in the treated than non-treated fracture sites. On histological analysis, fracture sites treated with FGF 2 showed a greater vascularity, periosteal reaction and cellularity. These studies suggest that fibroblast growth factors have properties which enhance fracture healing.

2.5. Summary

Osteogenesis is a complex process involving the differentiation of bone marrow stromal stem cells into osteoblasts with the formation of mineralised matrix. The behaviour of osteoprogenitors is influenced by several growth factors which have been shown to have specific effects on cellular activity in vivo and in vitro. The process is further influenced by cell – matrix and cell – cell interactions. Local tissue specific signals play a role in determining the subsequent differentiation of stromal stem cells implanted in skeletal sites. Investigation of the effects of osteogenic substances on the growth and differentiation of bone marrow stromal stem cells is important for the development of tissue engineering techniques to regenerate skeletal tissues.

Colloss is derived from bovine bone and contains several bone matrix associated growth factors. The availability of a variety of such factors may confer an advantage by allowing the stimulation of osteogenesis at several intermediate stages of osteoprogenitor differentiation. Preliminary studies investigating its osteogenic potential have been promising, but further investigation is required to obtain further data in relation to its effect at the cellular level, and its potential for use in clinical orthopaedic practice.

3. Chapter 1

Investigation of the Effects of Colloss on the Osteoblastic Differentiation of Human Bone Marrow Stromal Stem Cells

3.1. Introduction

Tissue engineering techniques utilising BMSSCs have been investigated as a means of regenerating, replacing and repairing musculoskeletal tissues. To date MSCs have been used to enhance tendon repair, fill cartilage defects and stimulate bone formation within skeletal defects with variable success.[111,112-114, 25] The processes involved in the differentiation of BMSSCs along lineage pathways is incompletely understood, but is believed to be regulated by complex intra and extracellular signalling pathways. [115,99, 116,117] The formation of mineralised matrix, following the differentiation of BMSSCs into osteoprogenitors has been demonstrated in tissue culture systems. Osteopontin, alkaline phosphatase and procollagen are expressed during osteogenic lineage progression of BMSSCs and can be used as markers of osteogenic differentiation. [95,118,119]

The osteoinductive properties of various growth factors have been examined using BMSSC culture systems. Substances which have been shown to be osteoinductive include the bone morphogenic proteins and fibroblast growth factor.[116,120]

Colloss (Collagen lyophilisate)

Colloss (Collagen lyophilisate) is a sterile extract of bovine osteoid matrix which contains collagen type 1. It is available in a solid form which has a cotton wool consistency and as a liquid suspension. It contains several cytokines which act at various steps in the cascade of osteogenic differentiation. In clinical studies, the solid

preparation has been used to fill and stimulate bone formation within phalangeal cysts and maxillofacial defects. To date no studies have been published which examine the effects of collagen lyophilisate on bone marrow stromal stem cells.

3.2. Aim and Hypothesis

Aim - To investigate the ability of Colloss to stimulate the osteogenic differentiation of adult bone marrow stromal stem cells (BMSSCs) *in vitro*.

Hypothesis - Colloss can stimulate the osteogenic differentiation of bone marrow stromal stem cells (BMSSCs) *in vitro*.

3.3. Materials and Methods

3.3.1. Harvest of bone marrow and isolation of stromal stem cells

Approval for this procedure was obtained from the Royal National Orthopaedic Hospital Ethics Committee and informed consent was obtained from the human bone marrow donor. Bone marrow was aspirated from a donor who was under general anaesthesia for an elective orthopaedic surgical procedure. The skin overlying the iliac crest was prepared and draped, then through a short skin incision bone marrow was aspirated using a bone marrow aspiration needle attached to a 10mm heparinised syringe.[121] All bone marrow stromal stem cells used in this study were derived from a single donor.

Mesenchymal stem cells were isolated from the bone marrow using the Ficoll Density gradient technique.[122,78] Bone marrow was loaded onto Ficoll-Hypoaque® (Amersham Pharmasin Biotech 75285, density gradient 1.077g/ml) within a universal container and centrifuged at 1000g for 30 minutes. Buffy coats containing the BMSSC enriched low density fraction were isolated and resuspended in medium comprising Dulbecco's modified Eagles medium (4500mg/L glucose/L, L-glutamine and sodium

pyruvate, Sigma Aldrich D6429), with 1% Penicllin / Streptomycin. (Sigma-Aldrich Co Ltd, Irvine UK) and 10% bovine foetal calf serum (FCS) and centrifuged for 5 minutes at 2000g. Supernatant was removed and pelleted cells were resuspended in medium by 3 successive aspirations and expulsions through a 23-gauge needle attached to a 5 millilitre syringe. Cells were counted using a Neubauer counting chamber and plated at a density of $2x10^5$cells/cm^2 into culture flasks (Falcon, Becton Dickinson Lab ware, Europe). Cells were then cultured with medium and incubated at 37°C in 95% air with 5% carbon dioxide at 100% humidity.

3.3.2. Cell Passage

Once cells had reached approximately 80% confluence, they were subcultured by the following steps. Culture medium was removed and trypsin (sterile filtered solution. 25g porcine trypsin per litre in 0.9% NaCl. Sigma-Aldrich Co Ltd, Irvine UK) was added to the culture flask, which was then incubated for 5 minutes at 37°C in 95% air and 5% carbon dioxide at 100% humidity resulting in detachment of cells. The flask was then rinsed with 5 mls of medium twice and rinsings were transferred to a universal container and centrifuged at 2000 rpm for 5 minutes. Supernatant was removed and pelleted cells were resuspended in 2mls of medium. A further 8 mls of medium was added to the cell suspension, which was then transferred into 175 cm^2 culture flasks (Falcon, Becton Dickinson Lab ware, Europe) and supplemented with medium. Cells were expanded in culture by serial passage on reaching confluence. Cells between passage 5 and 7 were used for all experiments.

Total DNA, proliferation, Alkaline Phosphatase activity, Collagen type 1(procollagen carboxy terminal peptide) and Osteopontin were measured in cells which were cultured in 6 well plates. For these experiments $3x10^4$ mesenchymal stem cells were seeded into wells at a density of 3000 cells/cm^2 . Each well was then filled with

2mls of standard medium (control group) or 2mls of medium containing collagen lyophilisate (Colloss dispersion, concentration 1.4d.m./ml, Ossacur AG) diluted 1:50 or 1:100. All cultures were performed in triplicate.

3.3.3. Characterisation of bone marrow stromal stem cells with STRO-1 IgM monoclonal antibody.

In this study the presence of Stro –1 cell surface marker was used to confirm that cell cultures comprised a multipotential bone marrow stromal stem cell population. STRO-1 is a cell surface antigen expressed by a subpopulation of bone marrow stromal cells capable of differentiation along several lineage pathways. Previous studies support the use of STRO-1 antigen as a stem cell surface marker. It has been demonstrated that bone marrow stromal cells positive for the STRO-1 antigen maintain an immature pre-osteoblastic phenotype and have the ability to differentiate into a variety of stromal cell types including adipocytes, chondroblasts and chondrocytes.[123,124]

Cells expressing the Stro –1 cell surface marker were identified using an indirect enzyme linked immunofluorescent antibody method. $3x10^4$ bone marrow stromal stem cells were seeded onto sterile cover slips (Falcon ^{TM}). One coverslip with cells was placed into each well of a six well culture plate. Wells were supplemented with control medium containing Dulbecco's modified Eagles medium (1000mg glucose/L, L-glutamine, $NaHCO_3$ and pyridoxine.HCL), with 1% penicillin / streptomycin. (Sigma-Aldrich co LTD, Irvine UK) and 10% bovine foetal calf serum (FCS). At 36 hours cells were washed 5 times with Dulbecco's phosphate buffered saline (Sigma D8537). Cells were then incubated in medium containing STRO-1 monclonal antibody (IgM mouse anti STRO-1, University of Iowa, Iowa city, IA 52242, USA.) diluted 1:20 for 1 hour at 37 °C in 95% air and 5% carbon dioxide at 100% humidity. Medium was then removed from the wells and cells were given a further 5 washes with phosphate

buffered saline, before incubation for 1 hour with anti mouse IgM-FITC monoclonal antibody (F9259 Sigma-Aldrich Co. Ltd, Irvine, UK) at a concentration of 1:128 in control medium. After the incubation period cells were mounted onto slides and observed using an Olympus BH-2 – RFCA microscope under fluorescent light.

3.3.4. Histological staining of mesenchymal stem cells in monolayer

3.3.4.1. Alizarin red

Mesenchymal stem cells were seeded onto 6 well plate culture dishes at a seeding density of $3x10^3$ cells/cm^2. In the control group, each dish was supplemented with 2 mls of standard medium comprising Dulbecco's modified Eagles medium (1000mg glucose/L, L-glutamine, $NaHCO_3$ and pyridoxine.HCL), with 1% penicillin / streptomycin. (Sigma-Aldrich Co Ltd, Irvine UK) and 10% bovine foetal calf serum (FCS).

In two experimental groups, the standard medium was replaced with medium supplemented with collagen lyophilisate at concentrations of 1:50 and 1:100. Cultures were incubated at 37°C in 95% air and 5% carbon dioxide at 100% humidity and stained for the presence of mineralised matrix with Alizarin Red on days 4, 8, 16 and 21 by the following procedure, which has been described by Stanford.[125] Medium was removed from the wells and the cell layer was washed with Dulbecco's phosphate buffered saline (Sigma-Aldrich Co. Ltd, Irvine KA12 8NB, UK) and then fixed by the application of 70% ice cold alcohol for 1 hour. Cells were then rinsed with distilled water five times and washed for 15 minutes in phosphate buffered saline to remove non-specific stain. Cultures were then air dried and photographed using a Nikon FE camera with a Nikon 60mm macro lens and 35mm Fujichrome 64 T Type II professional color reversal film ISO 64/19°.

3.3.4.2. Alkaline Phosphatase

Sterile 22mm x 22mm glass coverslips (Chance Propper Ltd, Smethwick, Warley, England) were placed in the wells of a 6 well culture plate. $3x10^4$ BMSSCs were seeded onto each coverslip and 2mls of either standard medium or medium containing Colloss 1:50 or 1:100 was placed in each well. Cells were stained for the expression of Alkaline Phosphatase using a method described by Stutte. [126]

The incubating media was prepared as follows: a buffer was prepared by the addition of 10mls of 0.2M Tris to 0.1M HCL made up to 40mls with distilled water. The pH of the buffer solution was then adjusted to 9.0 by the addition of 0.1M Hydrochloric acid as required. The incubating medium was completed by the addition of 10mg Napthol AS B1-Phosphate, 8 drops of 4% new fuschin in 2M HCL and 8 drops of 4% sodium Nitrite.

At 4, 8, 16 and 21 days medium was removed from the culture wells and cell layers were rinsed with phosphate buffered saline, following which cells were fixed with 10% formal saline. The incubating medium was pipetted onto the incubating dish and incubated at 37° C for 30 minutes. Incubating medium was then removed and coverslips were washed in distilled water for 2 minutes, rinsed and drained. Methyl green counter stain was added for 2 minutes. Coverslips were then air dried and mounted with pertex coverslip mounting medium. The presence of cells expressing Alkaline Phosphatase is signified by pink staining of cells.

3.3.5. Cell proliferation assay using alamar™ blue Assay

Alamar blue was used to measure cell proliferation. It is a dark blue dye containing an oxidation – reduction (REDOX) indicator, which when taken up by cells becomes reduced to a red colour which fluoresces. The amount of fluorescence can be quantified by measuring absorbance. The degree of conversion relates to the viability of cells and

their ability to metabolise alamar blue. Its measurement can therefore give a measure of cell number and over time a measure of cell proliferation. A linear relationship between fluorescence and cell number has been shown and some authors have found it to be comparable to thymidine incorporation assays for measurement of cell proliferation.[127, 128]

Cellular proliferation was measured using a technique adapted from Nakayama et al, 1997.[129] Cell proliferation was measured on days 3, 9, 14 and 21 by the following technique. Medium was removed from the wells of the 6 well plate and replaced with 2 millilitres of alamar blue diluted 1:10 in the cell media. Cells were then incubated for 4 hours at 37° C, humidity 100%, 5% CO_2. Two 100µl aliquots were then removed from each well and transferred to a 96 well plate.

Absorbance was measured at 595nm with a reference wavelength of 655nm using a Biorad microplate reader (model 3550, Bio-Rad Laboratories Ltd , Hemel Hempstead, Hertfordshire HP27TD). Analysis was performed in triplicate cultures. Culture of cells was continued by removing the remaining alamar blue contained medium from the wells and replacing it with either standard medium in the control groups or collagen lyophilisate containing medium in the experimental groups.

3.3.6. Total DNA using Hoescht 33258

Total DNA was measured on days 3, 7, 14 and 21 using a technique described by Rago et al, 1990.[130] The fluometric dye, Hoechst 33258, is DNA specific and binds to contiguous adenine-thymine base pairs emitting fluorescence at a wavelength of 460nm.

3.3.6.1. Cell lysis using freeze thaw cycle

Medium was removed from culture plate wells of the experimental and control groups and replaced with 2 mls distilled water. Cell lysis was achieved by a freeze thaw cycle. The culture plate containing cells was cooled to –70° C for 20 minutes followed by 20 minutes in a warm room at 37° C for 20 minutes. This cycle was repeated once.

3.3.6.2. Preparation of a standard curve

DNA 1mg/ml from calf thymus was used as a standard (Sigma D3664). This was diluted 1:50 in saline sodium citrate buffer to give a solution of concentration 20μg DNA per ml of buffer. This solution was sequentially diluted 1:2 to provide standards of 20, 10, 5, 2.5, 1.25, 0.63 and 0.31 μg/ml of buffer.

3.3.6.3. Preparation of Buffer

The buffer was made by adding 43.825grams of sodium chloride and 22 grams of Trisodium citrate to 480 mls of purified water. The pH was adjusted to 7.0 with 1M sodium hydroxide or 1M hydrochloric acid as required and the solution was made up to 500mls. Buffer was then sterilized by autoclaving. This buffer solution was diluted 1:20 for use in this procedure.

A 100µl sample of the distilled water used in the freeze – thaw cycle was transferred from each culture well plate in duplicate to the wells of a 96 well plate (Falcon TM). 100µl of Hoechst diluted to 1µl/ml of saline sodium citrate buffer was also added to each well. Fluorescence was measured at 460nm using a flurometer (Ascent reader, Labsystems). Analysis was performed using windows based Genesis software package.

3.3.7. Alkaline Phosphatase assay

Alkaline Phosphatase was measured on days 3, 7, 14 and 21 using a technique adapted from that described by Oreffo et al.[119] This method is based on the principle that the enzyme Alkaline Phosphatase cleaves the phosphate group from p-nitrophenol phosphate, resulting in the release of p-nitrophenol which has a yellow colour at alkaline pH and can be monitored at 405nm. The Cobas Bio analyser (Roche diagnostics, Welwyn Garden City, UK) calculates the results using the reaction rate method by which a series of readings are taken and plotted against time to provide a slope, which indicates the rate of appearance of the colour which correlates with Alkaline Phosphatase activity.

Medium was removed from wells and replaced with 2mls of distilled water. Cells were lysed to release ALP using the freeze-thaw cycle described previously. A 50µl sample of distilled water was taken from each well, from which ALP activity was analysed using the Cobas Bio. Precinorm U (Roche Diagnostics Ltd, Lewes, East Sussex UK) control serum used for calibration and was analysed with each assay to ensure quality control.

3.3.8. Calcium mineral content using Alizarin Red-S assay.

Calcium mineral content of cell layers grown in control medium and medium containing Colloss concentration 1:50 was measured with an Alizarin red-S assay using methods described by Stanford et al, 1995.[125] Alizarin red – S (AR-S) is a dye which binds selectively to calcium salts. Each 1 mol of AR-S binds to 2 mols of Ca^{2+}.

Bone marrow stromal stem cells were seeded into $25cm^2$ culture flasks (Falcon, Becton Dickinson Lab ware, Europe) at a density of 30,000 cells per flask. One group was supplemented with standard control medium and the other with standard medium containing Colloss diluted 1:50. Calcium mineral content was measured on days 7, 15 and 30 in triplicate cultures.

At the specified time periods, cultures were rinsed with PBS (Dulbecco's Phosphate buffered saline, Sigma – Aldrich Co Ltd, Irvine KA2 8NB,UK) and then fixed in 70% ice cold ethanol for a minimum of 1 hour. Cultures were then washed with distilled water and stained with 40mM Alizarin Red – S for 15 minutes followed by 5 further washes with distilled water. To remove non specific AR-S stain, cultures were washed in PBS for 15 minutes. The AR-S assay was performed by destaining the cell cultures by adding 1ml of a solution of 10% (W.V) Cetylpyridium chloride (CPC) in 10 mM sodium phosphate, pH 7.0, for 15 minutes at room temperature. AR-S extracts were then diluted 10-fold in the 10% CPC solution. 100µl from each sample was transferred to a 96 well plate and absorbance measurements were made at 595nm (reference 655nm) using a Biorad microplate reader (model 3550, Bio-Rad Laboratories Ltd , Hemel Hempstead, Hertfordshire HP27TD). The concentration of AR-S in each sample was calculated using an AR-S standard curve, with the microplate reader logistic curve fitting program. The concentration of calcium for each sample was

determined from the AR-S values. Experiments were performed in triplicate for each group.

3.3.9. Osteopontin immunoassay

Bone marrow stromal stem cells were seeded onto 6 well plates ($10cm^2$) at a seeding density of 3000 cells per cm^2. Cells were grown in either control medium or medium containing collagen lyophilisate diluted 1:100 or 1:50. Cells were grown for periods of 14 and 21 days. All medium exchanged during medium changes and collected at completion of the growth period was stored at –30° C. Following removal of medium, 1ml of distilled water was placed in each well and the cells were lysed by 2 freeze – thaw cycles; 20 minutes at –70° C and 20 minutes at 37° C. The lysate was used to determine the DNA content of the cell layer as discussed previously.

Osteopontin expression on days 14 and 21 was determined using a human osteopontin enzyme immunometric assay kit (TiterZyme ® EIA., Assay Designs, inc. 800 Technology Drive Ann Arbour, MI 48108 U.S.A). The optical density of samples was measured at 450nm (reference 590nm) with a Biorad microplate reader. A standard curve was established using standards comprising human osteopontin in concentrations of 640ng/ml, 320ng/ml, 160ng/ml, 80ng/ml, 40ng/ml, 20ng/ml, 10ng/ml and 5ng/ml. Analysis for calculation of results was performed with the Biorad microplate reader logistic curve fitting program.

3.3.10. Type I Procollagen radio immunoassay

Collagen type 1 is released from cells as a procollagen which has a carboxyterminal propeptide (PICP). The carboxyterminal propeptide is cleaved extracellularly to leave collagen type 1. Measurement of the carboxyterminal pro peptide of collagen type 1 allows quantification of type 1 collagen synthesis.[131,132]

In this study type 1 collagen was quantified using a type 1 procollagen radio immunoassay kit (Orion Diagnostica, Orion Corporation. PO Box 83, 02101 Espoo, Finland) utilising the radioisotope I ^{125}. A calibration curve was established using 6 standards comprising procollagen 0, 25, 50, 100, 200 and 500μg/l. The Type I Procollagen radio immunoassay was performed in BMSSC cultures grown in control medium or medium containing collagen lyophilisate at 7, 14 and 21 days.

The assay measures the concentration of the carboxyterminal propeptide of type 1 collagen. The assay is based on a competitive binding principle. A sample containing an unknown amount of procollagen carboxyterminal propeptide is mixed with a known quantity of radiolabelled procollagen. The labelled and unlabelled PICP then compete with antibody to PICP. When equilibrium is reached, the amount of radiolabelled antigen bound to antibody is inversely proportional to the amount of unlabelled PICP.

3.3.11. Statistics

The Kolmogorov – Smirnov test was used to determine the normality of data. On the basis of the Kolmogorov – Smirnov statistic, it was determined that variable data was non-parametric, hence comparisons of cell proliferation, DNA, Alkaline Phosphatase activity, osteopontin, collagen type I synthesis were made using the Kruskal Wallis test. Comparisons of Calcium content between colloss and control cultures was made using the Mann Whitney U test. Data analysis was performed using SPSS for Windows version 9.0.0 statistics software (Chicago, IL 60606).

3.4. Results

3.4.1. Mesenchymal stem cell characterisation using STRO-1 monoclonal IgM antibody

Thirty six hours after seeding, bone marrow cells were labelled with Stro-1 monoclonal antibody to detect the presence of the STRO-1 cell surface antigen, a stromal stem cell marker. (fig.1.)

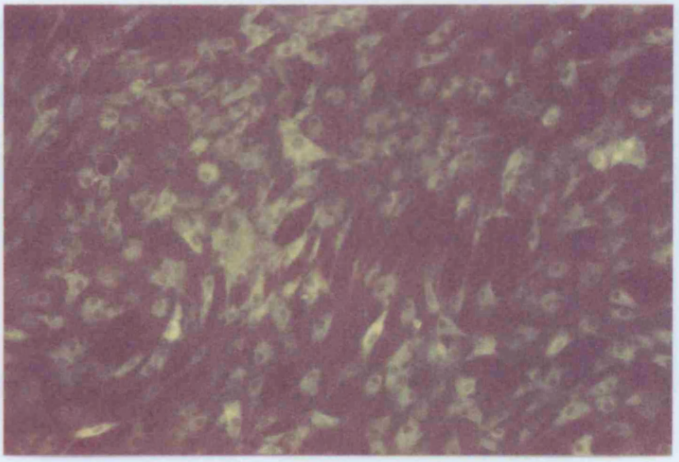


Fig.1. Enzyme linked immunofluorescence of bone marrow stromal stem cells in culture, labelled using anti STRO -1 IgM monoclonal primary antibody, 36 hours after seeding and observed with fluorescence microscopy. Magnification x 10.

3.4.2. Cell morphology

On day 1 of culture, BMSSCs in control and Colloss containing medium showed a fibroblastic phenotype typical of bone marrow stromal stem cells. At day 3, the majority of cells in the control group maintained a fibroblastic morphology (fig.2a), whereas many cells grown in the Colloss containing media developed a polyhedral morphology typical of osteoblasts. (figs2b and c). After 5 days in culture with Colloss, spindle shaped cells became sparse with the majority of cells showing polyhedral morphology typical of osteoblastic differentiation (figs3b and c). These cells appeared to account for a higher proportion of cells in the Colloss 1:50 than the Colloss 1:100 cultures. Control cultures maintained a fibroblastic phenotype (fig.3a).

At day 16 cells in both control and Colloss containing media had reached confluence. In contrast to cells in the control group those cultured in Colloss 1:100 and 1:50 developed multilayered cell aggregates (fig.4 a-c).

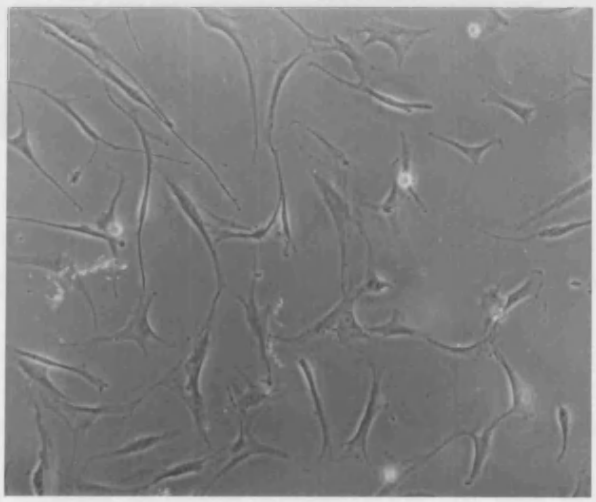


Fig.2a. Day 3 BMSSCs in control medium magnification X 10

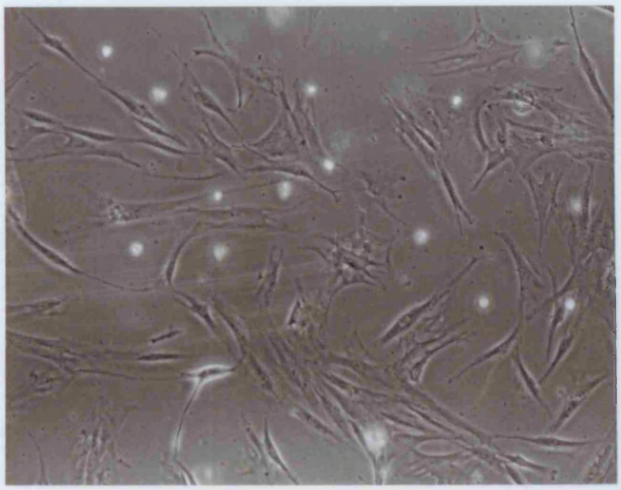


Fig.2b. Day 3. BMSSCs cultured in Colloss 1:100 magnification X 10.

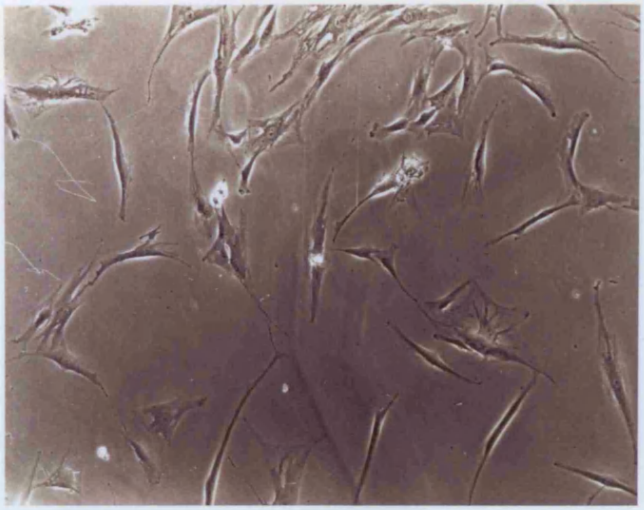


Fig.2c.Day 3. BMSSCs cultured in 1:50 Colloss magnification X 10.

Fig.2. Day 3 Photomicrographs of BMSSCs cultured in control medium (a) and medium containing Colloss at 1:100 concentration (b) and 1:50 concentration (c). Colloss containing media showing the presence of cells with a polyhedral morphology, suggesting osteoblastic differentiation.

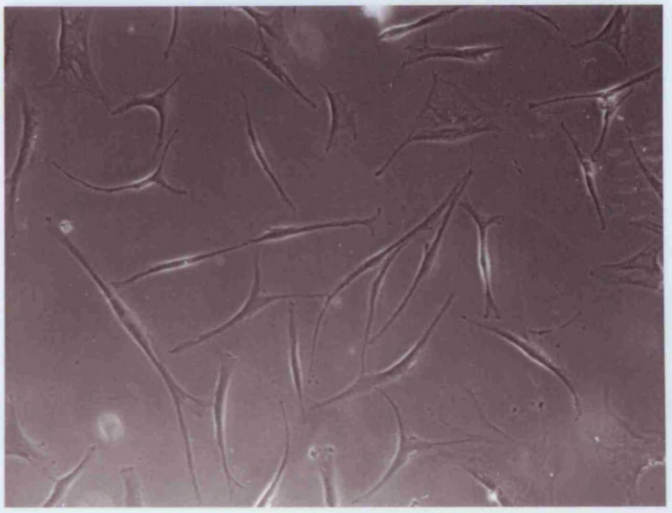


Fig.3a. Day 5. BMSSCs cultured in control medium. Magnification X 10

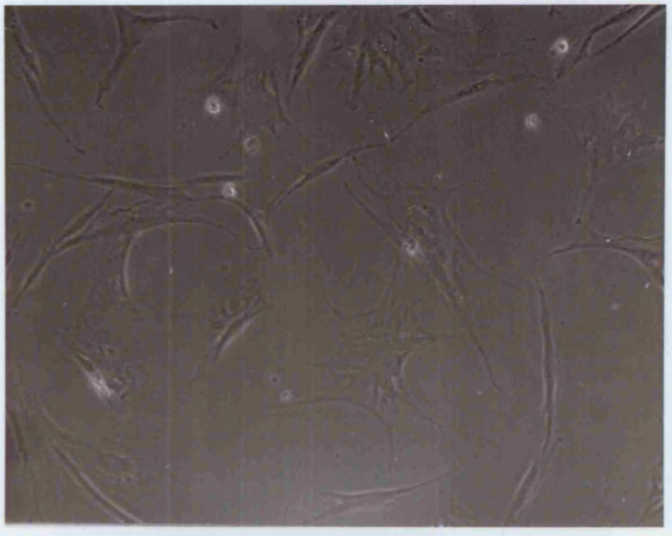


Fig.3b. Day 5. BMSSCs cultured in Colloss 1:100. Magnification X 10.

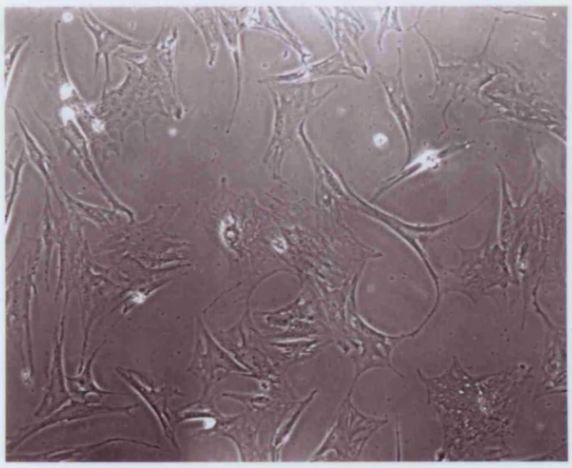


Fig.3c. Day 5 cultures. BMMSCs cultured in Colloss 1:50. Magnification X 10.

Fig.3 a, b and c. By day 5 BMSSCs in the control group (a) maintain their typical fibroblastic appearance whereas some of the cells grown in Colloss 1:100 (b) and most cells cultured in the Colloss 1:50 (c) media have developed a polyhedral osteoblastic morphology.

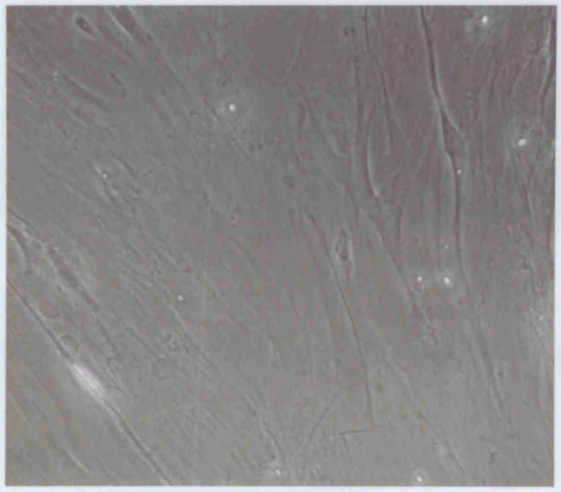


Fig.4a. DAY 16. BMSSCs cultured in control media. Magnification X 10

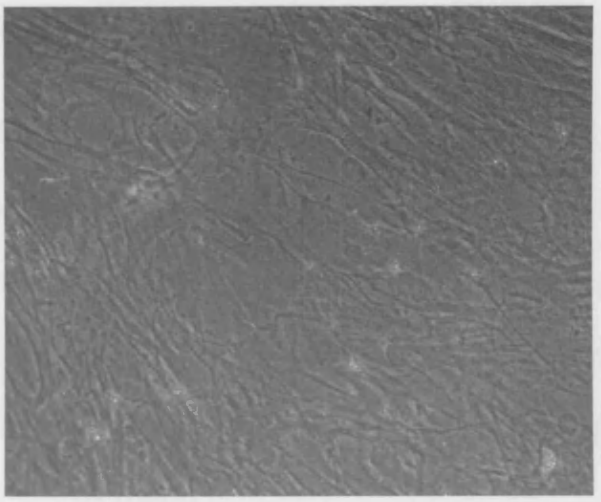


Fig.4b. Day 16. BMSSCs cultured in Colloss 1:100 media. Magnification X 10.

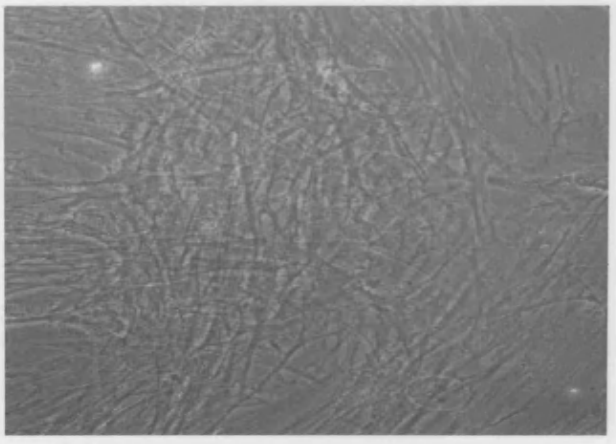


Fig.4c.Day 16 BMSSCs cultured in Colloss 1:50 media. Magnification X 10.

Fig.4. a, b and c. Day 16. Cells in all culture groups had reached confluence by day 16. In contrast to the control cultures (a), the cells grown in Colloss formed complex cell aggregations which were more prominent in the Colloss 1:50 (c) than the Colloss 1:100 group (b).

3.4.3. Cell Proliferation

Colloss 1:100 and 1:50 vs control media

A comparison of cell proliferation between cells grown in media containing Colloss concentration 1:100, 1:50 and cells grown in control media is shown in figure 5. On day 1 proliferation rate was greater in Colloss 1:100 cultures than Colloss 1:50 or control cultures (p=0.001). BMSSCs cultured in Colloss 1:50 had a higher proliferation rate than control cultures (p=0.010).

In all groups there was a significant increase in cell proliferation between days 1 and 3 (control p=0.04, Colloss 1:100 p=0.002, Colloss 1:50 p=0.004). On day 3, cells grown in Colloss 1:50 media had a significantly higher proliferation rate than those cultured in control (p=0.001) or Colloss 1:100 media (p=0.001). Cells cultured in control media had a higher proliferation than those in Colloss 1:100 media (p=0.001).

At day 9, cells cultured in Colloss 1:100 had a higher proliferation rate than those cultured in control or Colloss 1:50 medium (p=0.001) BMSSCs cultured in control medium had a higher proliferation than those grown in Colloss 1:50 medium (p=0.001). There was a significant decrease in proliferation rate in cells cultured in Colloss 1:50 and control media between days 3 and 9 (p=0.004), whilst cells cultured in 1:100 Colloss showed an increase in proliferation rate (p=0.004).

At day 14, in all groups there was a significant fall in proliferation rate between days 9 and 14 (p=0.004). At this time, cells cultured in Colloss 1:50 had a higher proliferation rate than those cultured in control and Colloss 1:100 medium (p=0.001). Cells cultured in control medium had a higher proliferation rate than those grown in Colloss 1:100 (p=0.001). By day 21, there was no difference in the proliferation rate between control and Colloss 1:50 groups. Cells cultured in Colloss 1:50 and control medium had a higher proliferation rate than those cultured in Colloss 1:100. (p=0.003).

Differences in proliferation rate between days 14 and 21 were significant for all groups ($p<0.01$).

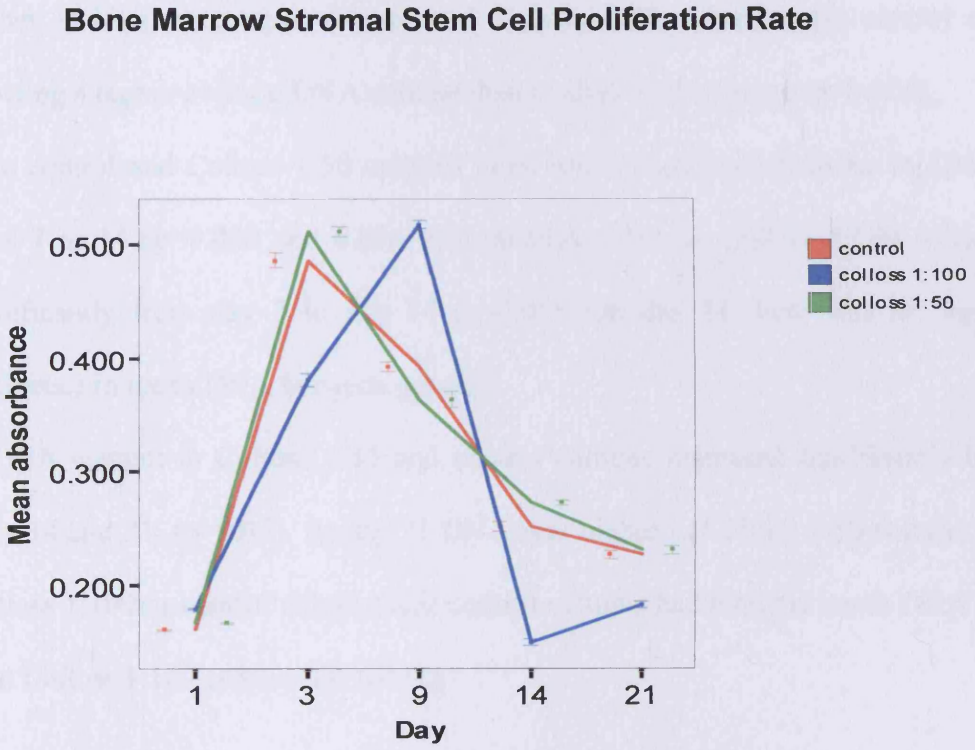


Fig.5. Graph of proliferation of cells grown in Colloss 1:50 and 1:100 vs control media
The error bars represent the standard error of the mean

3.4.4. DNA

At days 1 and 3 there was no significant difference in total DNA between cultures grown in control or Colloss containing media. At day 7 Colloss 1:100 cultures had a higher DNA content than control and Colloss 1:50 cultures with control cultures showing a higher average DNA content than Colloss 1:50 cultures ($p=0.027$).

In control and Colloss 1:50 cultures there was a significant increase in DNA from days 7 to 14 ($p=0.050$ and 0.046 respectively). DNA in Colloss 1:100 cultures fell significantly from day 7 to day 14 ($p=0.05$). On day 14 there was no significant difference in mean DNA between groups.

DNA content in Colloss 1:50 and control cultures increased significantly between days 14 and 21. ($p=0.05$). At day 21 DNA was higher in Colloss 1:50 cultures than in Colloss 1:100 or control cultures and control cultures had a higher mean DNA content than Colloss 1:100 cultures ($p=0.027$).

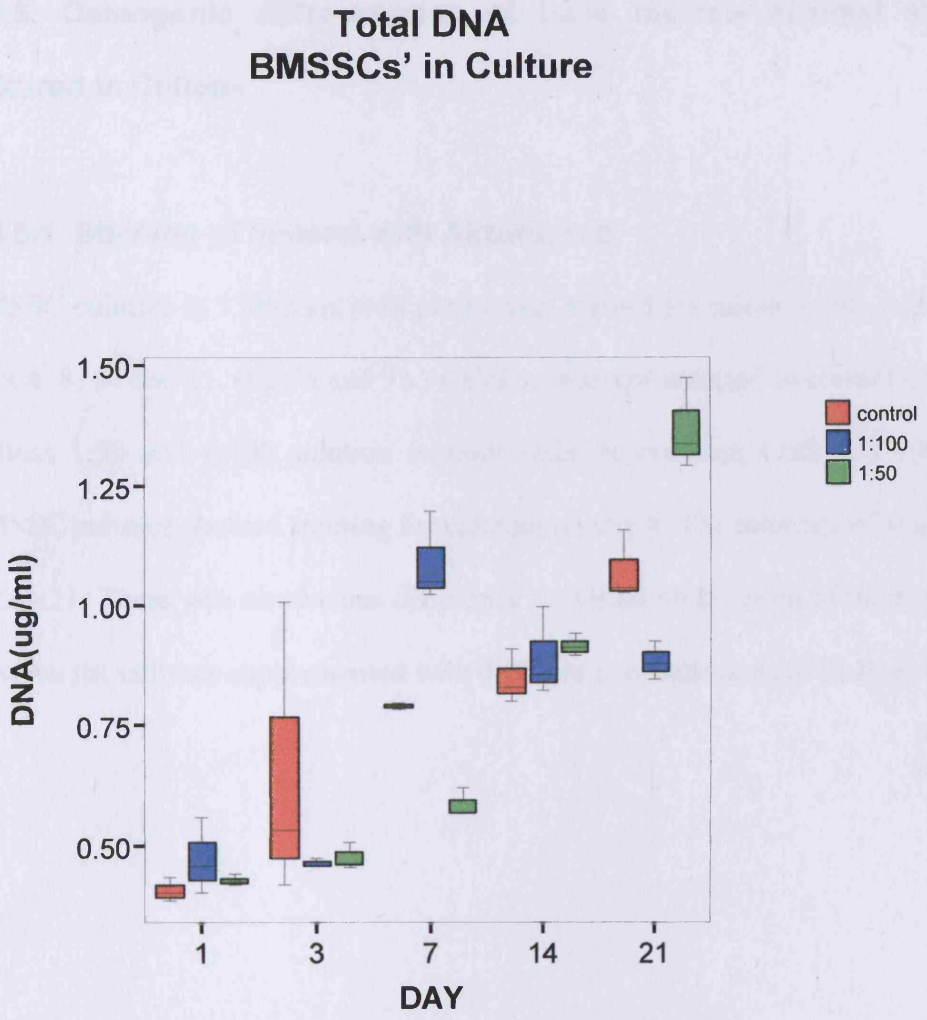


Fig.6. Boxplot of DNA Content per culture well.

3.4.5. Osteogenic differentiation of bone marrow stromal stem cells cultured in Colloss

3.4.5.1. Staining of mineral with Alizarin red

BMSSC cultures in 35mm six well plates were stained for calcium with Alizarin red on days 4, 8, 16 and 21. (fig.7a and 7b.) Calcium was not detected in control cultures or in Colloss 1:50 and 1:100 solution without cells. In contrast, Colloss 1:100 and 1:50 BMSSC cultures showed staining for calcium on day 8. The intensity of stain increased to day 21. There was no obvious difference on visual observation of the stain intensity between the cultures supplemented with different concentrations of Colloss.

Fig.7a. Staining of BMSSC cultures for calcium with Alizarin red on days 4, 8 and 16. Only the upper three wells in each plate were used for culture

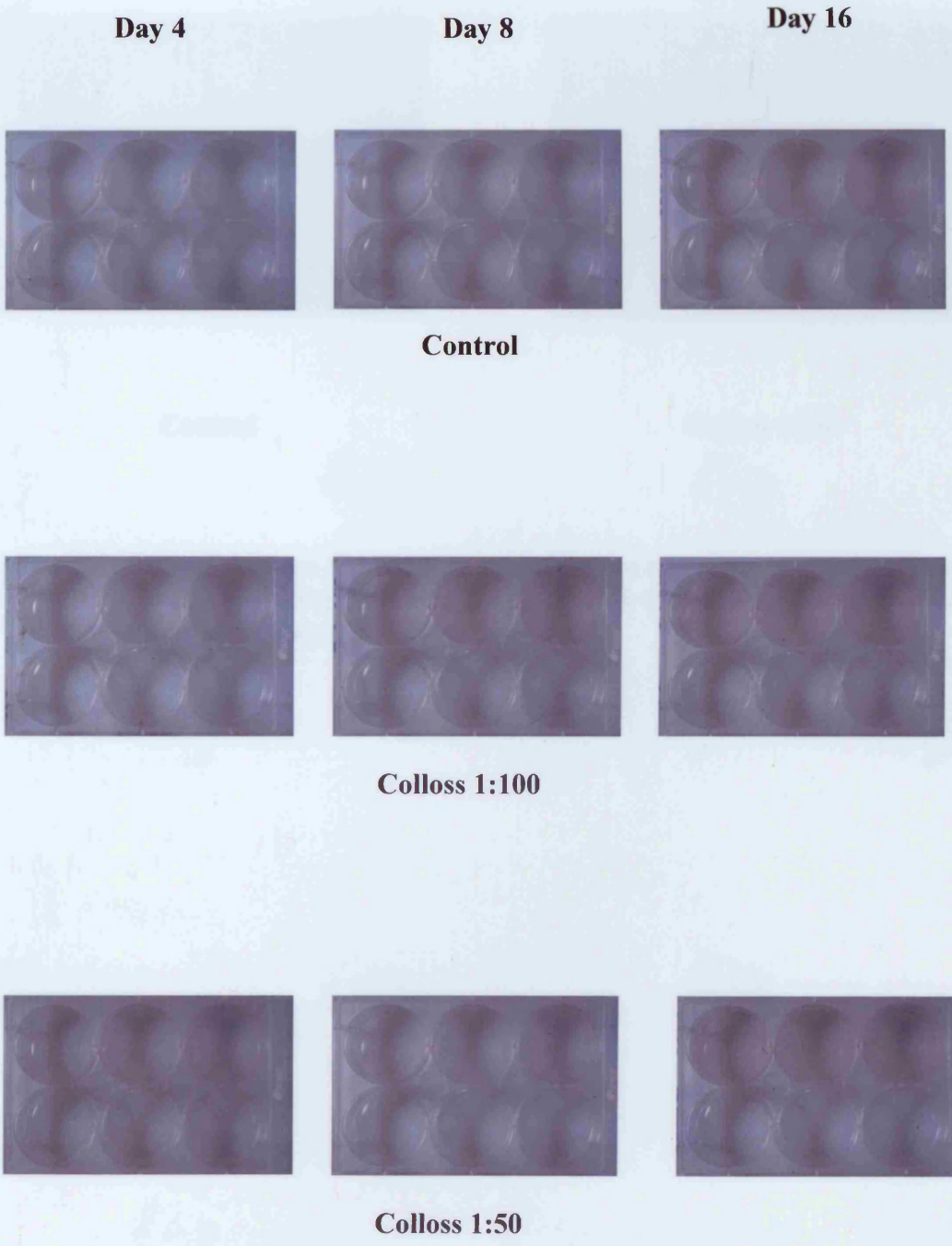
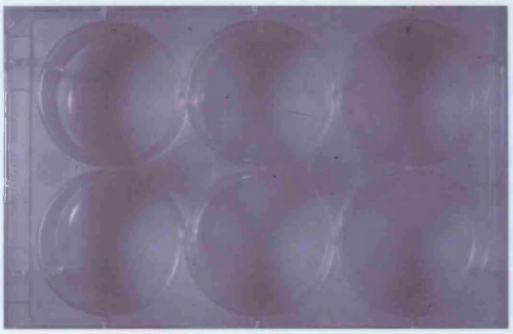
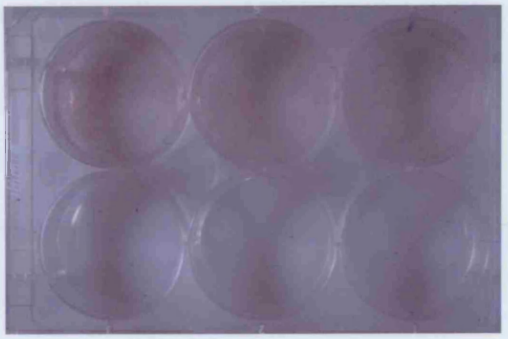


Fig.7b. Comparison of day 21 day cultures stained for calcium with Alizarin red. Control and Colloss 1:50 cultures

Day 21





Control **Colloss 1:50**

Figs 7a and b. Control cultures did not stain for calcium at any of the selected time periods up to day 21. Alizarin red stained calcium was detected in Colloss supplemented cultures on day 8 with the intensity of stain increasing to day 21.

3.4.5.2. CALCIUM

Calcium content was compared between control cultures and cultures with Colloss concentration 1:50. Before day 15, calcium was not detected in control or Colloss containing cultures. At day 15 calcium was detected in Colloss containing cultures. The calcium content in Colloss cultures increased from day 15 to day 30, but this increase was not significant (p=0.513). No calcium was detected in the control cultures for the duration of the cultures to day 30 (fig.8). These results indicate that Colloss 1:50 stimulates an increase in the calcium content of BMSSC cultures.

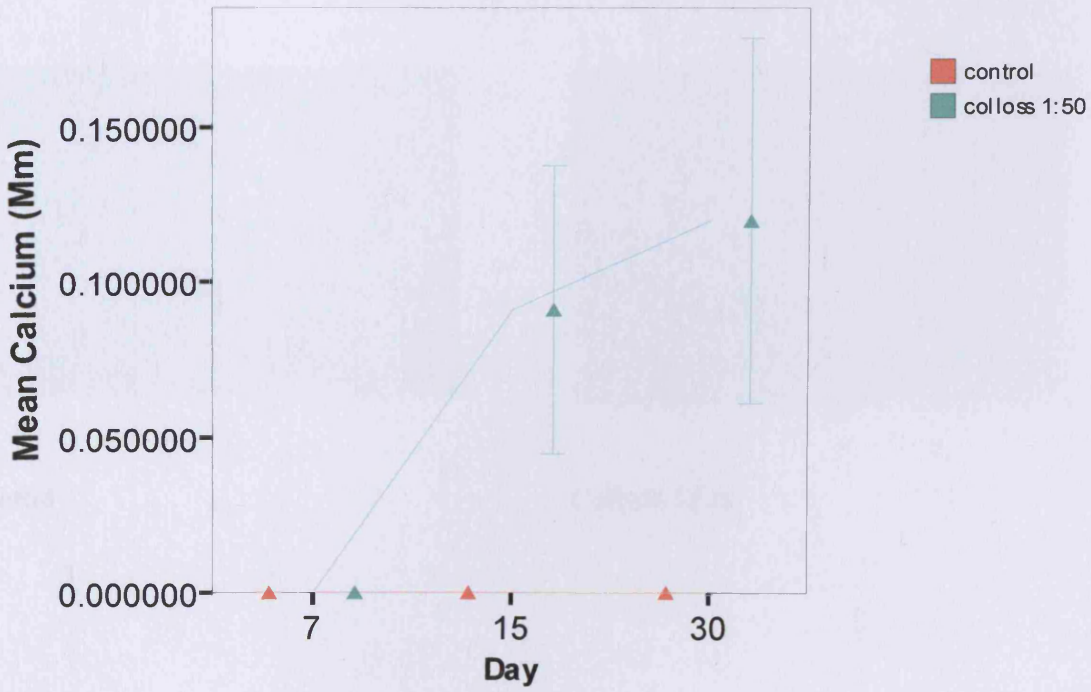


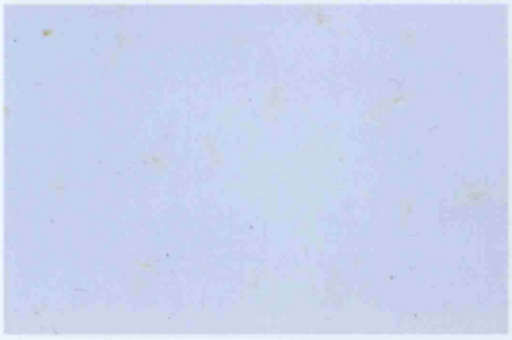
Fig.8. Mean calcium content per culture flask. Control vs Colloss 1:50
The error bars represent the standard error of the mean

3.4.5.3. Histological Staining of BMSSC Cultures for Alkaline Phosphatase Expression.

Alkaline Phosphatase expression by bone marrow stromal stem cells grown in control medium and medium containing Colloss on days 4, 8, 16 and 21 is shown in figures 9a-e with Alkaline Phosphatase positive cells staining pink.

Alkaline Phosphatase is expressed in the control and Colloss cultures. There appeared to be an increase in the number of cells positive for Alkaline Phosphatase in both control and Colloss groups as the duration of culture increased.

Fig.9a. Day 4



Control

Colloss 1:100

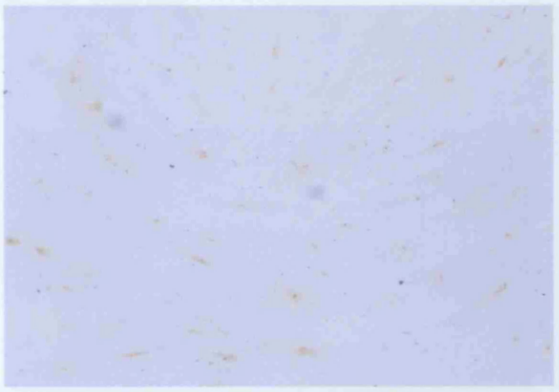
Fig.9b. Day 8

Control

Colloss 1:100

Colloss 1:50

Fig.9c. Day 12



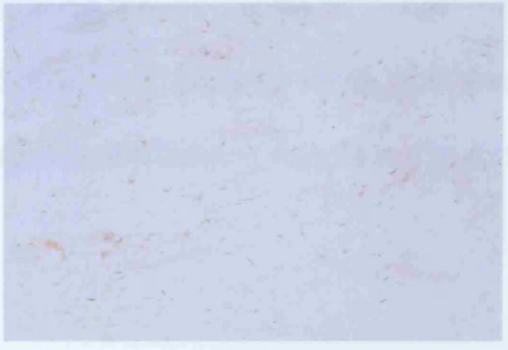
Control

Colloss 1:100

Fig.9d. Day 16

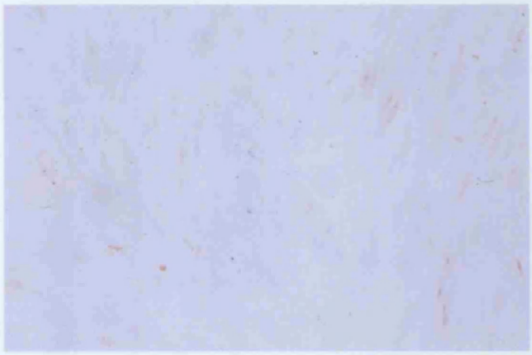
Control

Colloss 1:100



Colloss 1:50

Fig. 9e. Day 21



Control

Colloss 1:100

3.4.5.4. ALKALINE PHOSPHATASE ACTIVITY (COBAS BIO)

Alkaline Phosphatase expression is shown in fig.10. On days 7 and 14 there was no significant difference in ALP expression between groups. On day 21 Cultures with Colloss 1:100 and 1:50 expressed significantly more Alkaline Phosphatase than those in control cultures ($p=0.027$). In all groups Alkaline Phosphatase levels appeared to fall from day 7 to 14 but this difference was significant only in the Colloss 1:50 group and Colloss 1:100 group ($p=0.05$). In all groups Alkaline Phosphatase expression increased significantly between days 14 and 21 ($p=0.05$).

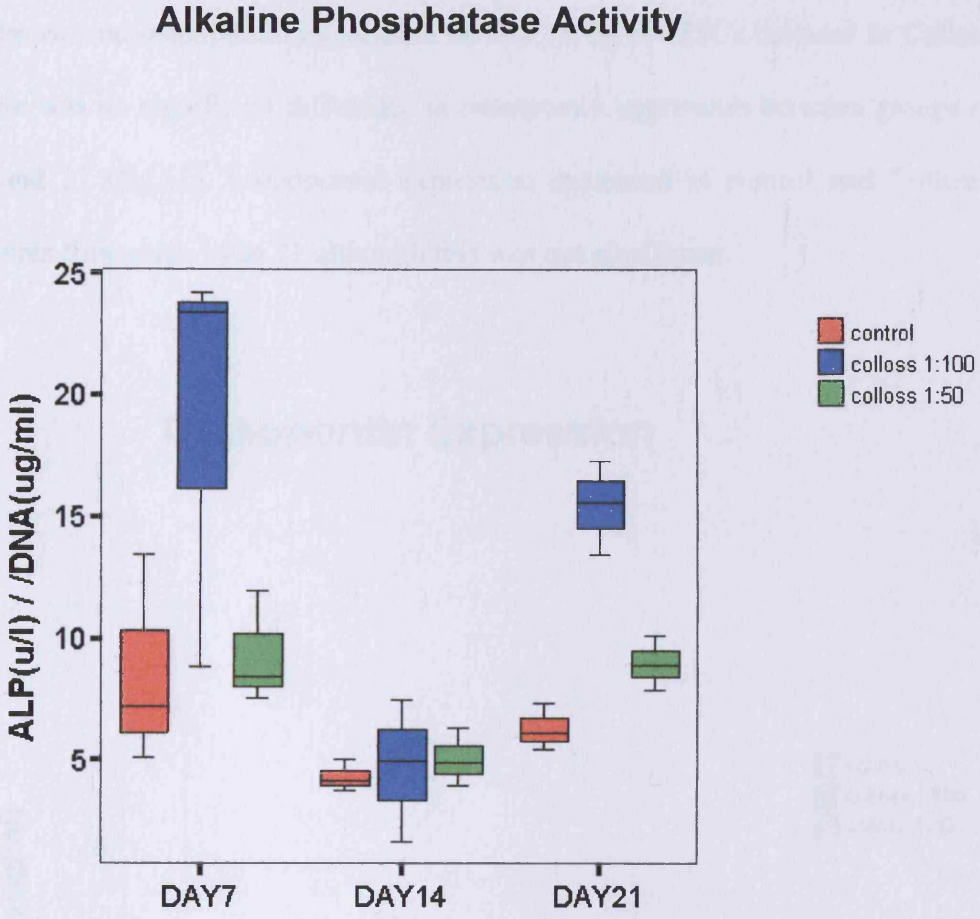


Fig.10. Boxplot of Alkaline phosphatase activity per culture well for BMSSC cultures. Control vs Colloss at two different concentrations

3.4.5.5. OSTEOPONTIN

There was no osteopontin expression on day 14 by BMSSCs cultured in Colloss 1:50. There was no significant difference in osteopontin expression between groups on days 14 and 21 (fig.11). Osteopontin expression decreased in control and Colloss 1:100 cultures from days 14 to 21 although this was not significant.

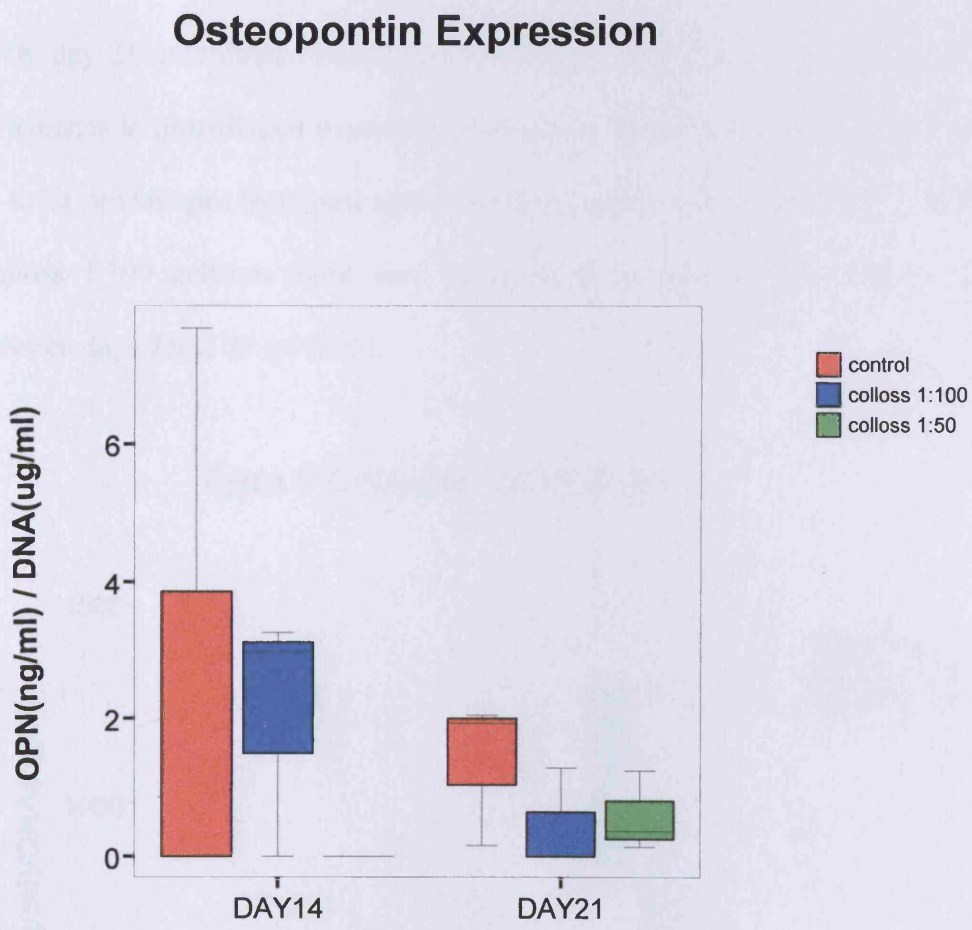


Fig.11. Boxplot of Osteopontin expression of BMSSC cultures per culture well.

3.4.5.6. Procollagen expression

On day 7 there was no significant difference in procollagen synthesis between Colloss and control cultures. There was a significant increase in Procollagen synthesis from day 7 to 14 only in Colloss 1:50 cultures (p=0.050). On day 14, There was no significant difference in procollagen between Colloss and control cultures(p=0.113). This difference was significant between control and Colloss 1:50 cultures (p=0.05).

On day 21 procollagen expression was highest in Colloss 1:100 cultures although, differences in procollagen expression between cultures were not significant. From day 14 to 21 procollagen increased significantly in control cultures (p=0.05). In control and Colloss 1:100 cultures there was a significant increase in procollagen expression between days 7 and 21 (p=0.05).

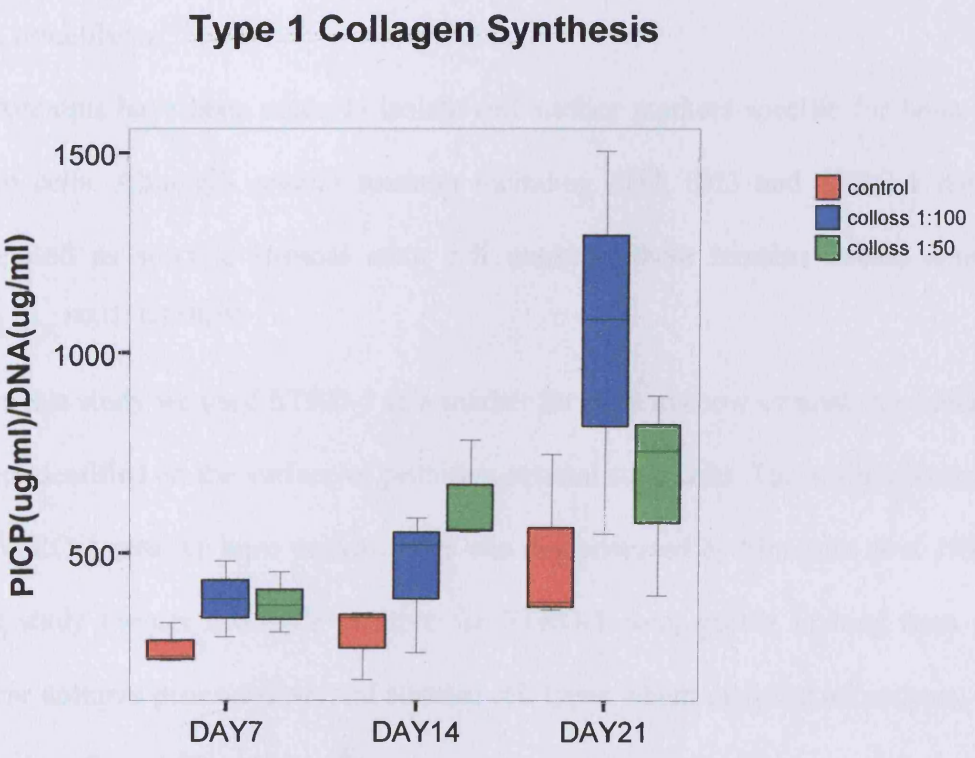


Fig.12. Boxplot of Type 1 collagen synthesis per culture well. Measured by quantification of Carboxyterminal pro-peptide.

3.5. Discussion

The findings of this study give some support to the hypothesis that Colloss stimulates the osteoblastic differentiation of bone marrow stromal stem cells *in vitro*. Some, but not all, of the markers of osteogenic differentiation were enhanced by the addition of Colloss to culture medium. The addition of Colloss to culture medium resulted in increased Alkaline Phosphatase expression, increased collagen type I expression and changes in cell morphology. Perhaps the most significant finding was that of calcium deposition in the Colloss containing cultures.

Bone marrow stromal stem cells express a multitude of cell surface markers which include integrins, growth factor and cytokine receptors, cell adhesion molecules and several miscellaneous antigens.[118] Many of these markers are also expressed by differentiated mesenchymal tissue cells such as adipocytes, fibroblasts, chondrocytes and osteoblasts.[133]

Attempts have been made to isolate cell surface markers specific for bone marrow stem cells. Although several markers including SH2, SH3 and STRO-1 have been proposed as specific stromal stem cell markers, there remains debate about their validity.[133,134,135,136,137]

In this study we used STRO-1 as a marker for bone marrow stromal stem cells. It has been identified on the surface of primitive stromal stem cells. The multipotential nature of STRO-1 positive bone marrow cells was demonstrated by Simmons et al 1991.[124] In this study human BMSSCs positive for STRO-1 were grown in long term culture. These cultures generated several stromal cell types which included adipocytes, smooth muscle cells and fibroblasts .[124]

In a further study Dennis et al 2002 demonstrated the multipotential properties of STRO-1 positive bone marrow cells by stimulating their differentiation into osteoblasts, chondrocytes, adipocytes and fibroblasts.[123] Although these studies provided evidence that STRO-1 positive cells can differentiate to form a variety of mesenchymal cell types, they have not demonstrated the multipotential ability of individual STRO-1 positive cells. This would require the stimulation of cloned STRO-1 cells to differentiate along the various lineage pathways.

Support for the validity of the STRO - 1 positive cells used in this study comes from the fact that another investigator in our institution stimulated differentiation of these cells into osteoblasts and adipocytes by adding osteogenic and adipogenic supplements to culture media.

The patterns of proliferation rate were similar in all cultures, with an early rapid proliferation phase to day 3 in Colloss 1:50 and control cultures and to day 9 in Colloss 1:100 cultures. This is followed by a rapid fall in proliferation rate from day 9 to 14 in all groups. Between days 14 and 21 there was a further decline in cell proliferation activity except for the Colloss 1:100 group where there was a moderate increase in cell proliferation.

As early as day 3, cells cultured in Colloss containing media, developed a polygonal phenotype typical of osteoblastic differentiation. At day 5 the majority of cells appeared to demonstrate this appearance. In contrast, cells grown in control media maintained spindle type morphology until confluence at day 16.

On subjective observation, there appeared to be a larger proportion of cells visualised under light microscopy with a polygonal appearance in the 1:50 Colloss group compared to the 1:100 Colloss group at day 5. However, confirmation would require more objective methods of quantification to determine whether increasing the concentration from 1:100 to 1:50 had a stimulatory effect on the differentiation of

human bone marrow stromal stems to express an osteoblastic phenotype. At day 16 cultures in control and Colloss containing media had reached confluence. Cells in the Colloss groups developed a complex multilayered configuration, which was not seen in control cultures.

The early phenotypic changes of bone marrow cells cultured in Colloss medium observed in this study are similar to those described by Jaiswal et al 1997,[78] who developed a culture system to examine the effect of osteogenic supplements on bone marrow stromal cells. When the osteogenic supplements dexamethasone, sodium β glycerophosphate and ascorbic acid were added to culture medium, BMSSCs were shown to develop an osteoblastic morphology as early as day 2, with cells later forming multilayered configurations.[78]

The ability of Colloss to stimulate the differentiation of bone marrow stromal stem cells along osteoblastic lineage pathways is further supported by the higher Alkaline Phosphatase activity levels found in the Colloss containing cultures on day 21. There was a stepwise increase in culture DNA content from day 1 through to peak levels on day 21 in the control and Colloss 1:50 cultures. In the Colloss 1:100 cultures, peak DNA content was measured on day 7.

Alkaline Phosphatase has a role in the mineralisation process during osteogenesis. In vivo it hydrolyses organic phosphates in the extracellular matrix, resulting in the release of phosphate which precipitates with calcium to form mineral apatite. Previous studies have examined Alkaline Phosphatase activity in bone marrow stromal cell cultures grown in osteogenic media. Majors et al, 1997, cultured human bone marrow stromal cells in media containing the osteogenic supplements β glycerophosphate sodium ascorbate and dexamethasone.[138] Alkaline Phosphatase was measured using a nitrophenyl phosphate ALP assay. Osteoblastic differentiation and mineralisation of

cultures was associated with Alkaline Phosphatase levels which increased between days 9 and 16 then remained relatively constant to day 30.

Jaiswal et al, 1997, examined the effects of osteogenic supplements on BMSSCs *in vitro.*[78] BMSSCs showed an increase in Alkaline Phosphatase activity to day 12 in both the control and osteogenic supplemented cultures. Cultures with osteogenic supplements had a higher ALP activity than the control cultures on days 4, 8, 12 and 16. ALP activity in the osteogenic supplemented stem cells peaked at day 12 then declined to day 4 levels on day 16.

In my study alkaline phosphase activity increased significantly from days 14 to 21 in both the control and Colloss cultures, and on day 21 was greater in the Colloss cultures than control cultures. Surprisingly there was no statistical difference in Alkaline Phosphatase activity between day 7 and day 21 in any of the groups.

The ability of Colloss to stimulate the mineralisation of stromal stem cell cultures provides further confirmation of its osteogenic capacity. There was evidence of matrix mineralisation at 8 days in Colloss cultures, whereas no calcium was detected in control cultures for the 30 day culture duration. The lack of mineralisation of stromal stem cell cultures in the absence of osteogenic supplements or growth factors has been shown in previous studies[78]. Mineralisation induced by Colloss occurred earlier than has been reported in studies where BMSSCs have been cultured with osteogenic supplements, when evidence of mineralisation can take up to 5 weeks.[87] However, the onset of mineralisation has varied between studies and Majors et al, 1997, reported matrix mineralisation at 17 days using Alizarin red to stain for calcium in cultures of bone marrow cell aspirates and Bruder et al 1998 observed mineralisation of matrix at 16 days using von kossa histochemistry to stain for calcium in BMSSCs cultured in osteoinductive supplemented media .[138,118]

Osteopontin is an extracellular protein expressed by osteoblasts which is involved in cell attachment during the process of bone formation and remodelling.[139] It has been shown to accumulate in the mineralised matrix of new bone.[140] More recently the expression of osteopontin by osteoclasts has been demonstrated.[141] There is evidence that osteopontin can enhance osteoclast motility and ability to resorb bone.[142] Despite its expression by osteoclasts[143], it has been used as a marker of osteogenesis in several studies [144, 145, 146]. Bruder et al, 1998, examined the expression of osteopontin mRNA in a bone marrow stromal cell culture system.[118] It was expressed maximally in the late phase of osteogenesis.

At the 14 and 21 day time points chosen to measure oseopontin, no difference was noted between control and Colloss containing cultures. It is possible that day 21 was too early in the osteogenic process, and that longer culture periods are required before maximal expression of osteopontin occurs. We found that osteopontin was expressed in the control BMSSCs untreated with Colloss. This has been demonstrated in previous studies where BMSSCs have been cultured *in vitro* in the absence of osteogenic supplements.[118,147] Although osteopontin is expressed by osteoblasts, its presence does not signify mineralisation. It is a fairly non specific marker of osteogenesis and its expression in the absence of mineralisation has been described.[148,149] Therefore, although the expression of osteopontin was not enhanced by Colloss, one cannot conclude that Colloss does not enhance osteogenesis.

Summary

Some, but not all, of the osteogenic markers measured in this study provided evidence suggesting that Colloss enhances the osteogenic differentiation of bone marrow stromal stem cells. Perhaps the most significant finding was the deposition of calcium in the Colloss treated specimens from day 15 onwards. Further evidence comes from the morphological changes noted in the Colloss treated specimens at an early stage.

4. Chapter 2

An Investigation of Growth and Differentiation of Bone Marrow Stromal Stem Cells Cultured on Smooth Polished Titanium Pre - Treated with Colloss

4.1. Introduction

Titanium is a biomaterial which is frequently used for the manufacture of orthopaedic prosthetic implants.[29,28,30] Titanium alloy has favourable characteristics which include its biocompatibility, corrosion resistance, light weight and the ease with which it can be machined by implant manufacturers.

Loosening of the femoral prosthesis remains a major factor limiting the longevity of total hip replacements. The loosening process is multi-factorial and is influenced by implant design, surgical technique and patient dependent variables. An important mechanism of the loosening process in total hip replacements is the formation of polyethylene wear particles from the acetabular component, which migrate along the interface between bone and implant and cement-bone interfaces. On reaching these interfaces they are phagocytosed by macrophages. This stimulates macrophages to release cytokines, resulting in the activation of osteoclasts, leading ultimately to periprosthetic bone resorption.[150] Eventually a fibrous membrane forms at the bone implant interface.[151] This fibrous layer compromises biological bonding between the femoral stem and host bone, leading to an increase in motion between the implant and host, thus exacerbating the loosening process.

Current methods of improving the osseointegration of femoral stems include: the provision of flutes to improve the initial anchorage of un-cemented femoral stems to host bone; modification of surface topography of the implant by shot blasting and porous coating with titanium or hydroxyapatite (HA).[152,153,154,155] Hydroxyapatite has

been shown to be effective in enhancing the osseointegration of coated femoral stems. [27,156] It has osteoconductive properties, enhancing the ingrowth of osseous tissue at the implant surface, but its osteoinductive capacity is limited. Furthermore, in the longer term, HA coatings are susceptible to delamination and degradation. [64,157,158]

Cell interactions at the implant – host interface are important and govern the osseointegration process. The osseointegration process involves the formation of bone from the endosteal surface of the medullary cavity which grows towards the implant.[61,159] Bone deposition may also occur at the implant surface following the deposition of a bone like apatite layer.[160,161]

Bone marrow stromal cells are primitive cells which have the ability to differentiate along lineage pathways to form bone, fibrous tissue and adipose tissue. They are present within the medullary cavity and are involved in the process of new bone formation on cemented implants. The growth, attachment and differentiation characteristics of bone marrow stromal cells on titanium surfaces has been investigated in previous studies. Titanium surface characteristics have been shown to influence cell adhesion, proliferation and differentiation. Deligianni et al 2001 found that increasing the roughness of titanium surfaces resulted in an increase in human BMSSC adhesion and proliferation.[162] With regards to differentiation of BMSSCs along osteogenic pathways, Ter Brugge et al, 2002[163], found that osteocalcin was expressed to a greater degree on smooth rather than rough surfaced titanium, but Castellani et al, 1999, was unable to demonstrate any effect of surface roughness on cell differentiation. [164]

Surface treatment of titanium by various methods has been shown to influence cellular morphology, proliferation and differentiation. Such treatments include topographical modification, calcium phosphate coating, alkali and heat treatment. [52, 165,166,167] Titanium forms an oxide layer on exposure to air, which provides the excellent corrosion resistive properties of titanium alloys. If this layer is compromised, there will

be an increase in the release of metal ions. There is evidence to suggest that metal ion release can influence osteoblast behaviour. Ching-Hsin et al, 2002, examined proliferation and ALP activity from osteoblasts grown on titanium which had undergone ageing treatment to reduce metal ion release and non-treated titanium.[168] Osteoblasts grown on treated titanium demonstrated a higher proliferation rate at 72 hrs and expressed a peak ALP level later than the control group.

The coating of implants with substances which have osteoinductive properties may provide a means of utilising the bone regenerative capacity of BMSSCs within the medullary cavity of the femur to improve osseointegration of femoral stem prostheses. The differentiation of such cells along osteogenic lineage pathways and the formation of mineral apatite on implant surfaces provide a mineralisation front, stimulating new bone formation which may improve osseointegration of metallic implants.

Colloss has osteoinductive properties. It contains cytokines which have stimulatory effects at several intermediate stages in the cascade of osteogenesis. This study will investigate the ability of Colloss to stimulate proliferation and osteogenic differentiation of bone marrow stromal stem cells growing on titanium biomaterials.

4.2. Aim and Hypothesis

Aim - To investigate the the effect of Colloss on the proliferation and differentiation of BMSSCs cultured on titanium biomaterials

Hypothesis – Colloss will enhance the osteogenic differentiation of bone marrow stromal stem cells cultured on smooth polished titanium

4.3. Materials and Methods

4.3.1. Preparation of Titanium Implants

Titanium alloy disks (Titanium318 TiV4AL6, Titanium International Ltd. Keys House Granby Ave, Birmingham B33 OSP, England) of diameter 5mm were "smooth polished" using silica alumina paste (Buehler alpha micro polish 1 Alumina, 41 Waukegan Rd. Lake Bluff. IL60044 USA). They were then immersed transiently in 70% alcohol followed by rinsing with distilled water. Disks were autoclaved at 126 degrees centigrade (11 minute sterilising time) using a Prestige Medical, series 2100, clinical autoclave. (P.O. Box 154 Clarendon Road, Blackburn, Lancs, BB1 9UG. UK).

4.3.2. Application of Colloss to Titanium Implants

Titanium disks were placed in the wells of a 24 well tissue culture plate. Colloss coated disks were prepared by pipetting 50 µl of collagen lyophilisate suspension (Colloss dispersion, concentration 1.4d.m./ml, Ossacur AG, Oberstenfeld, Germany) on to the surface of each titanium disk in the experimental group. In the control group titanium disks were also placed in 24 well culture plates but were not coated with Colloss suspension. Disks were then left undisturbed for a period of one hour prior to the seeding of bone marrow stromal stem cells.

4.3.3. Seeding of Titanium Disks with Bone Marrow Stromal Stem Cells

Bone marrow stromal stem cells were isolated and cultured as described in Chapter 1(3.3.1). Each titanium disk was placed in the well of a 24 well culture plate. Thirty thousand ($3x10^4$) cells were seeded onto each smooth polished titanium disk using a micro pipette. After seeding of bone marrow stromal stem cells, the 24 well plates containing titanium disks were incubated at 37°C in 95% air and 5% carbon dioxide at

100% humidity. After 30 minutes of incubation, 2 mls of standard medium comprising Dulbecco's modified Eagles medium (4500mg/L glucose/L, L-glutamine and sodium pyruvate, Sigma Aldrich D6429), with 1% Penicillin / Streptomycin. (Sigma-Aldrich Co Ltd, Irvine UK) and 10% bovine foetal calf serum (FCS) was added to each well containing titanium disks. One day after cell seeding, the medium was discarded and titanium disks were transferred to another 24 well tissue culture plate (Day 0). This was performed to exclude cells which may have migrated onto the tissue culture plastic of the 24 well plate after seeding. Medium changes were performed at selected intervals, at the same time for the control and Colloss coated titanium disks.

4.3.4. Cell Proliferation Using Alamar™ Blue Assay

The alamar blue assay provides a means of quantifying cell proliferation. It contains a REDOX indicator that fluoresces and changes colour in response to cellular proliferation. Colour change can then be quantified by measuring absorbance.

Cell proliferation was measured on days 1, 3, 9, 14 and 21 using an alamar blue assay, utilising techniques described in chapter 1(3.3.5). At the selected time intervals medium was removed from culture wells and replaced with 2mls of a solution containing Alamar blue 1:10 in standard medium. Cells were then incubated for 4 hours at 37° C, humidity 100%, 5% CO_2. After the four hour incubation period, two 100 µl aliquots of medium were then removed from each well and transferred to a 96 well plate.

Absorbance was measured at 595nm with a reference wavelength of 655nm using a Biorad microplate reader (model 3550, Bio-Rad Laboratories Ltd , Hemel Hempstead, Hertfordshire HP2 7TD). Analysis was performed in triplicate cultures. Culture of

cells was continued by removing the remaining alamar blue containing medium from the wells and replacing it with standard medium.

4.3.5. Total DNA Measurement

Total DNA was measured on days 7, 14 and 21 using the fluorometric DNA binding dye, Hoescht 33258, as described in chapter 1(3.3.6.). At selected time intervals medium was removed from the culture plate wells containing the titanium disks and replaced with 1ml of distilled water. Lysis of cells to release DNA was performed by 2 freeze thaw cycles, freezing cells to –70° C for 20 minutes followed by warming to 37° C for 20 minutes. DNA from calf thymus was used for the preparation of a standard curve, as described in chapter 1 (3.3.6.2).

One hundred microlitres of cell lysate and DNA standard was transferred to a 96 well plate in duplicate (Falcon™, Becton, Dickinson, NJ, USA). One hundred microlitres of Hoechst 33258 diluted to 1µl/ml of saline sodium citrate buffer was also added to each well. Fluorescence was measured at 460nm using a flurometer (Ascent reader, Labsystems Inc, 8 East Forge Parkway, Frankin, MA 02038, USA). Analysis was performed using Genesis software package.

4.3.6. Alkaline Phosphatase assay

Alkaline Phosphatase was measured on days 7, 14 and 21 using the technique previously described in chapter 1(3.3.7.). Culture medium was removed from wells of the 24 well plate and replaced with 2mls of distilled water. Cells were lysed to release ALP with a freeze thaw cycle of freezing to –70° C for 20 minutes followed by 37° C for 20 minutes, which was performed twice. A 50µl sample of distilled water was taken from each well, from which ALP activity was analysed using the Cobas Bio analyser.

4.3.7. Type I Procollagen carboxyterminal propeptide radio immunoassay

Radioimmunoassay of the carboxyterminal propeptide of type 1 procollagen was used as a measure of type 1 collagen synthesis using the techniques described in chapter 1 (3.3.10). Type one collagen synthesis was measured from the culture medium in which the control and Colloss coated titanium disks were grown. All medium collected during medium changes and at completion of the growth period was stored at –30°.

Type I Procollagen in control titanium and Colloss treated titanium bone marrow stromal cell cultures was measured in culture medium on days 14 and 21 using a type 1 procollagen radioimmunoassay utilising the radioisotope I ^{125}(Orion Diagnostica, Orion Corporation. PO Box 83, 02101 Espoo, Finland). A calibration curve was established using 6 standards comprising procollagen 0, 25, 50, 100, 200 and 500µg/l.

4.3.8. Osteopontin immunoassay

Osteopontin was measured from the culture medium in which the control and Colloss coated titanium disks were grown using the technique described in chapter 1(3.3.9.). All medium exchanged during medium changes and collected at completion of the growth period was stored at –30° C. Osteopontin expression on days 14 and 21 was determined using a human osteopontin enzyme immunometric assay kit (TiterZyme ® EIA., Assay Designs, Inc. 800 Technology Drive Ann Arbour, MI 48108 U.S.A).

4.3.9. Scanning Electron Microscopy

Titanium and bone marrow stromal stem cell culture samples were prepared for electron microscopy at selected time intervals. Disks were fixed in 1.5% glutaraldehyde and 0.1 M sodium cacodylate buffer for 48 hours at 4 degrees. Samples were then washed in 0.2% sodium cacodylate for 1 hour and fixed in Osmium tetroxide 1% in 0.1 M sodium cacodylate buffer. Samples were then washed twice for 5 minutes in 0.2M sodium cacodylate and placed in 1% tannic acid in 0.05% sodium cacodylate for 1 hour. Samples were then washed with sodium cacodylate buffer 4 times (2 minutes each) and dehydrated in increasing concentrations of alcohol before air drying in hexamethyl disalazane. Samples were mounted onto electron microscopy stubs. Silver in methyl isobutyl ketone (Acheson electrodag 1415M) was applied to the sample – stub interface to prevent charging by the electron beam. Samples were then coated with Palladium using an Emitech K550 coating apparatus. Microscopy was performed using a JEOL (JSM-550LV) scanning electron microscope.

4.3.10. Statistics

On the basis of the Kolmogorov – Smirnov statistic it was determined that variable data was not normally distributed. Statistical comparison between treated and control samples for cell proliferation, DNA, Alkaline Phosphatase, osteopontin and collagen type I synthesis comparisons were made using the Mann Whitney U test.

4.4. Results

4.4.1. Cell proliferation rate

In both groups, proliferation rate increased significantly between days 1 and 3 (fig.1.) (p=0.004), at which time proliferation peaked in both groups. There was no statistical difference in peak proliferation rate between groups on day 3. From day 3 to day 9 proliferation rate decreased significantly in control cultures (p=0.004). Day 9 proliferation rate was significantly greater on cells grown on titanium pre-treated with Colloss than those in the control group (p=0.016). From days 9 to 14 proliferation rate decreased significantly (p=0.004) in the pre-treated titanium group and by day 14 proliferation was significantly lower than cells in the control group (p=0.010). From day 14 to 21 cell proliferation rate fell significantly in both Colloss and control groups (p=0.004).

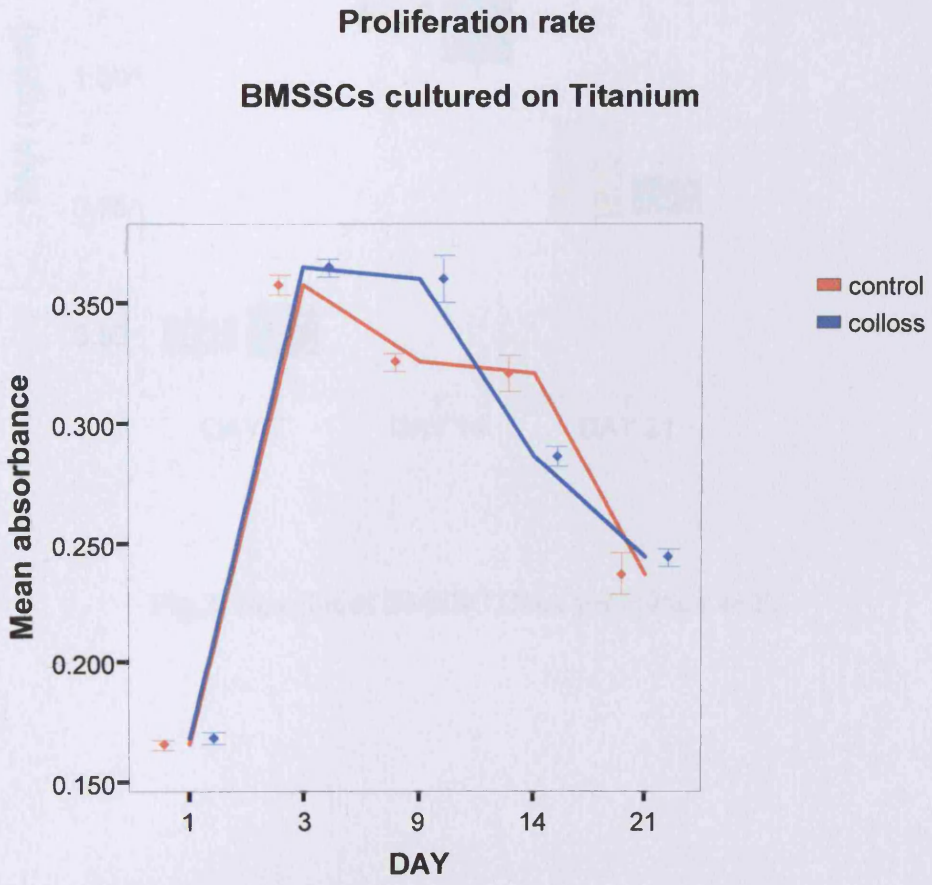


Fig.1. BMSSC proliferation rate

Error bars show mean +/- 1 standard error

4.4.2. Total DNA

There was no significant difference in total DNA in control and Colloss cultures on days 7, 14 and 21(fig.2.).

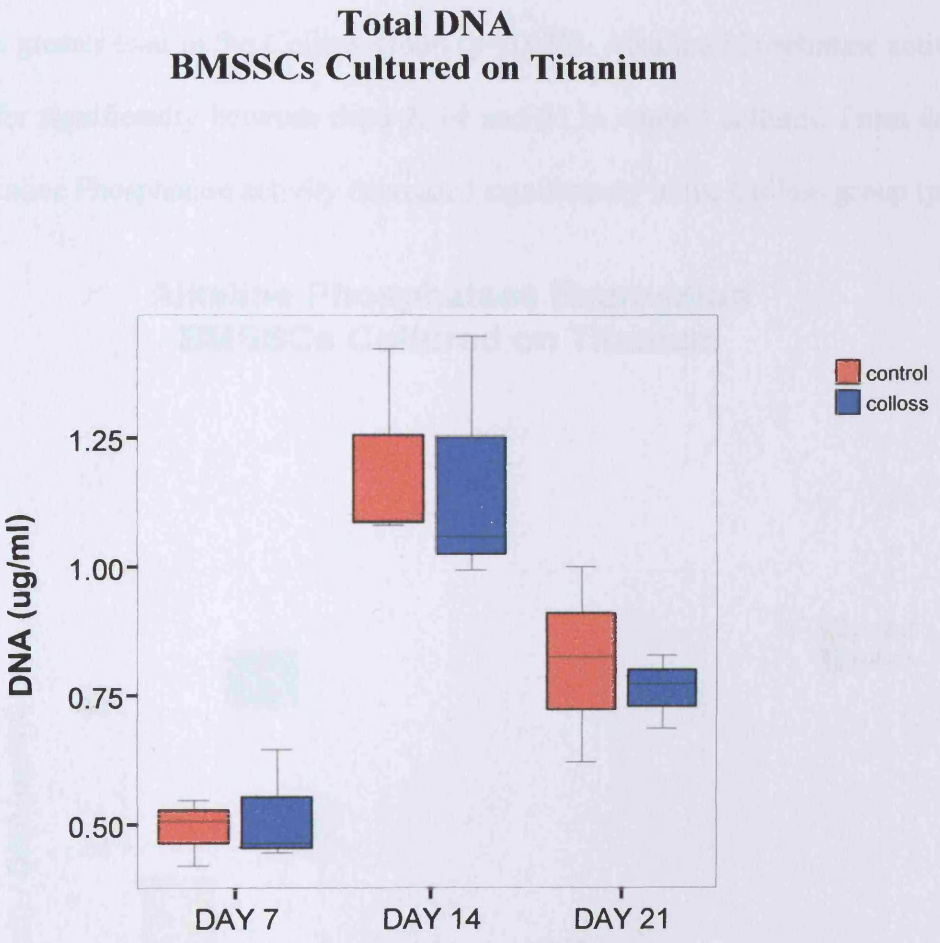


Fig.2. Boxplot of BMSSC DNA per culture well.

4.4.3. Alkaline Phosphatase Activity

On day 7 Alkaline Phosphatase activity was greater in the Colloss group than control cultures (p=0.050) (fig 3.). On day 14 there was no difference in ALP expression between control and Colloss cultures and on day 21 ALP activity in control cultures was greater than in the Colloss group (p=0.050). Alkaline Phosphatase activity did not differ significantly between days 7, 14 and 21 in control cultures. From days 7 to 14 Alkaline Phosphatase activity decreased significantly in the Colloss group (p=0.05).

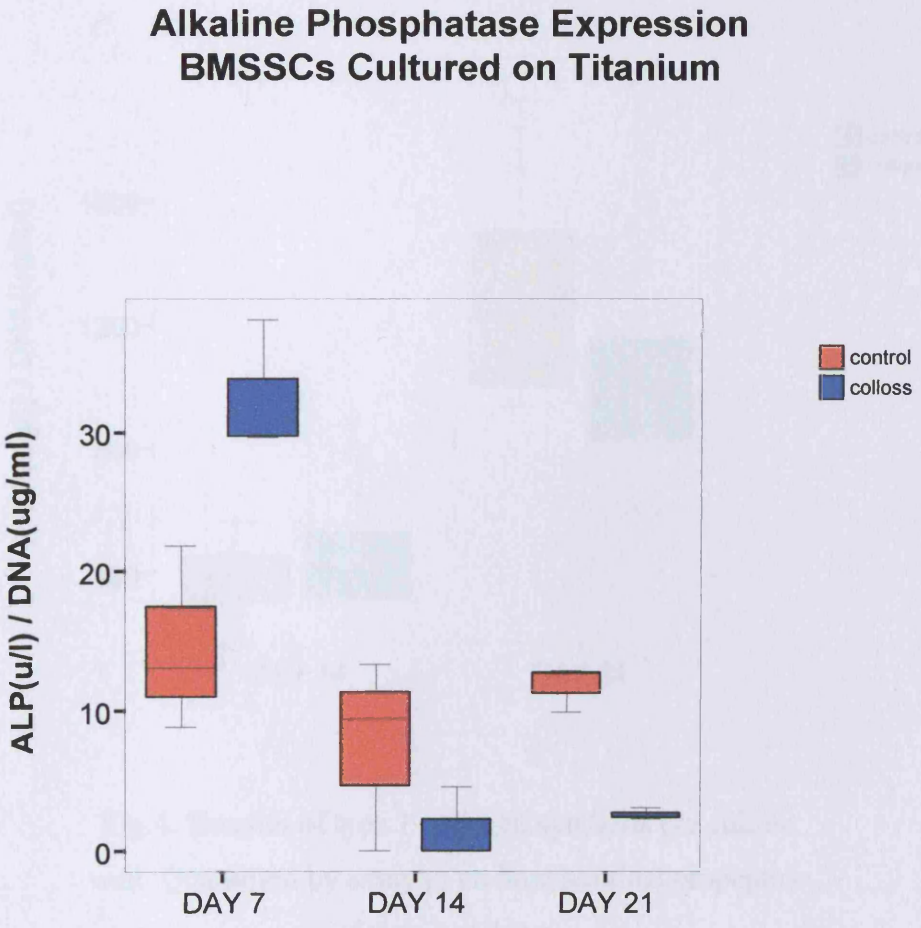


Fig.3. Boxplot of Alkaline phosphate activity per well.

4.4.4. Procollagen Expression

There was no difference in procollagen measured between cells grown on titanium treated with Colloss and BMSSCs grown on non-treated titanium on days 14 and 21 (fig.4). Procollagen expression increased significantly from days 14 to 21 only in control cultures (p=0.050).

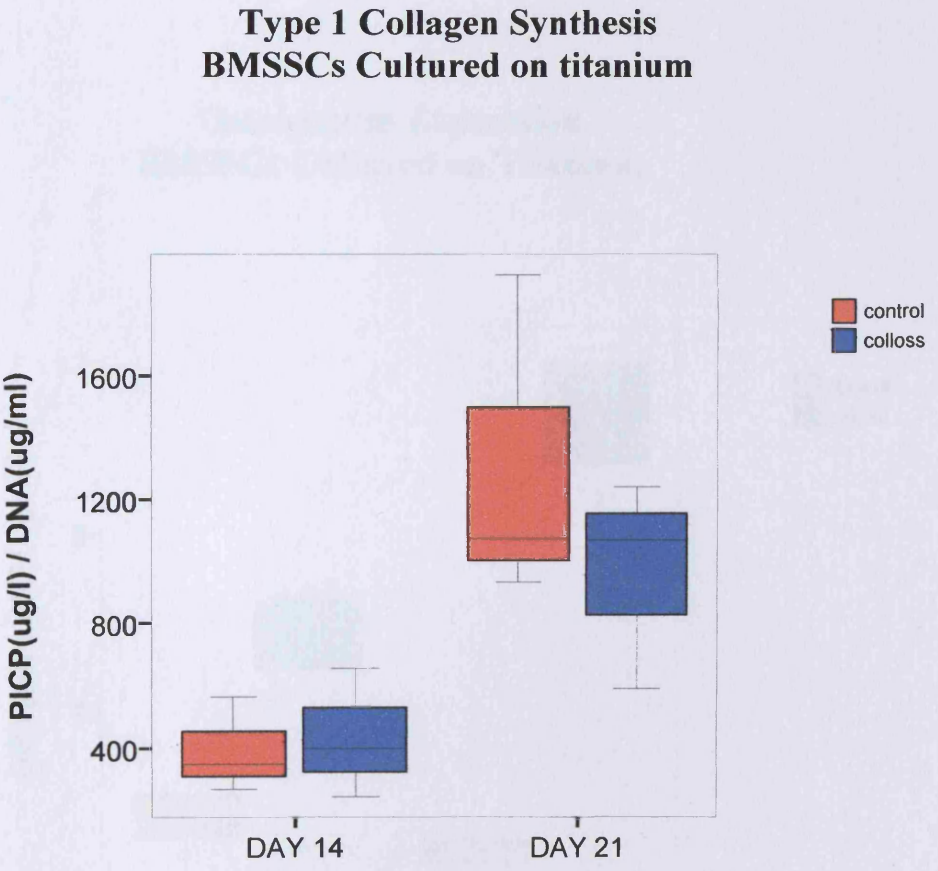


Fig.4. Boxplot of type 1 collagen synthesis per culture well. Quantified by assay of carboxyterminal propeptide of type 1 collagen.

4.4.5. Osteopontin expression

Osteopontin expression increased significantly from days 14 to 21 in the group pre treated with Colloss (P=0.05). On days 14 and 21 osteopontin expression was significantly greater in the group pre-treated with Colloss than the control group. There was no significant difference in osteopontin expression between control cultures on days 14 and 21 (fig.5.).

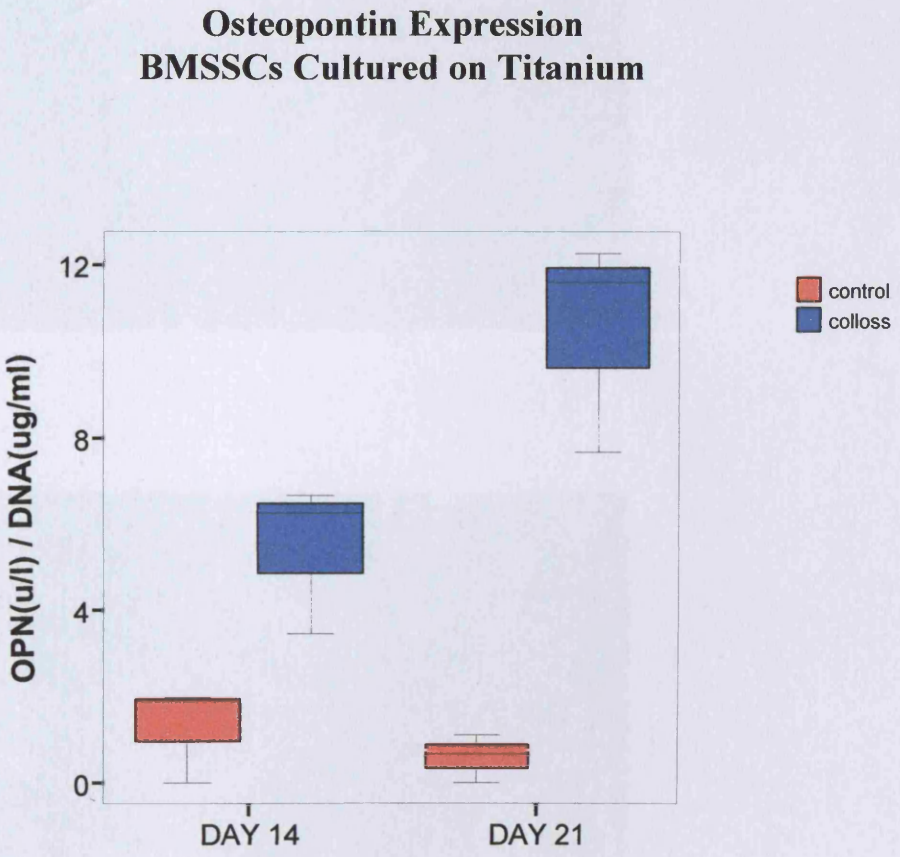
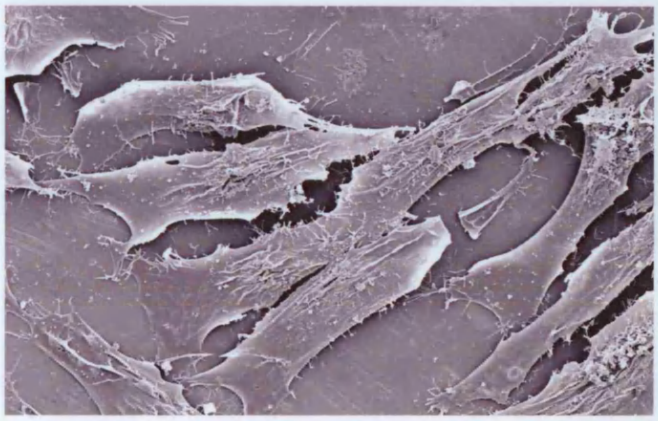


Fig.5. Boxplot of Osteopontin Expression per culture well.

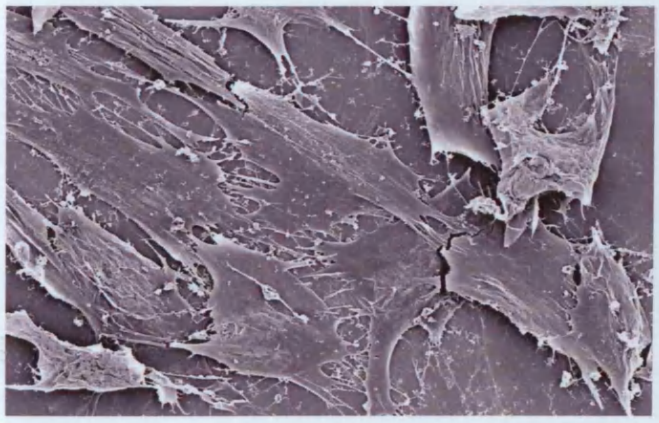
4.4.6. Scanning Electron Microscopy

Scanning electron microscopy was performed to determine the morphology of cells cultured on the titanium biomaterials. BMSSC morphology in control and Colloss groups is shown in figs 6, 7 and 8.

Fig.6a and b. Day 7. BMSCCs S.E.M. X 600.



a

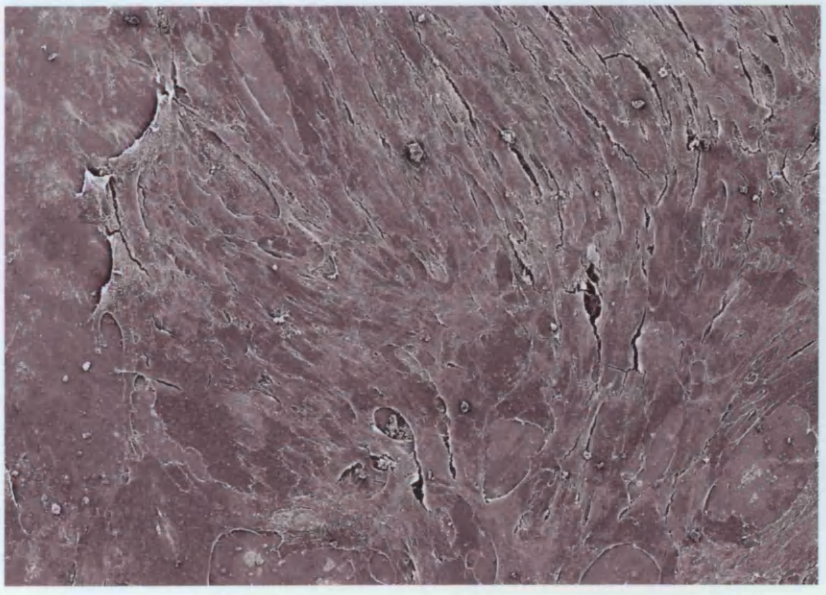


b

Fig.6. Control cells (a) and cells cultured on titanium pre treated with Colloss (b).

Fig.7a and b. Day 14. BMSSCs. SEM x 200. (A) control; (B) Colloss

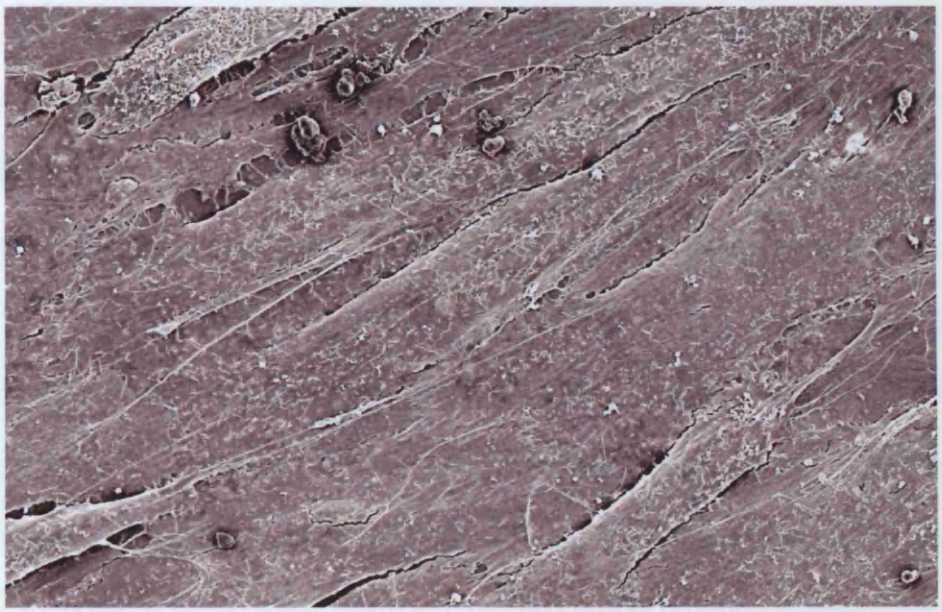
a



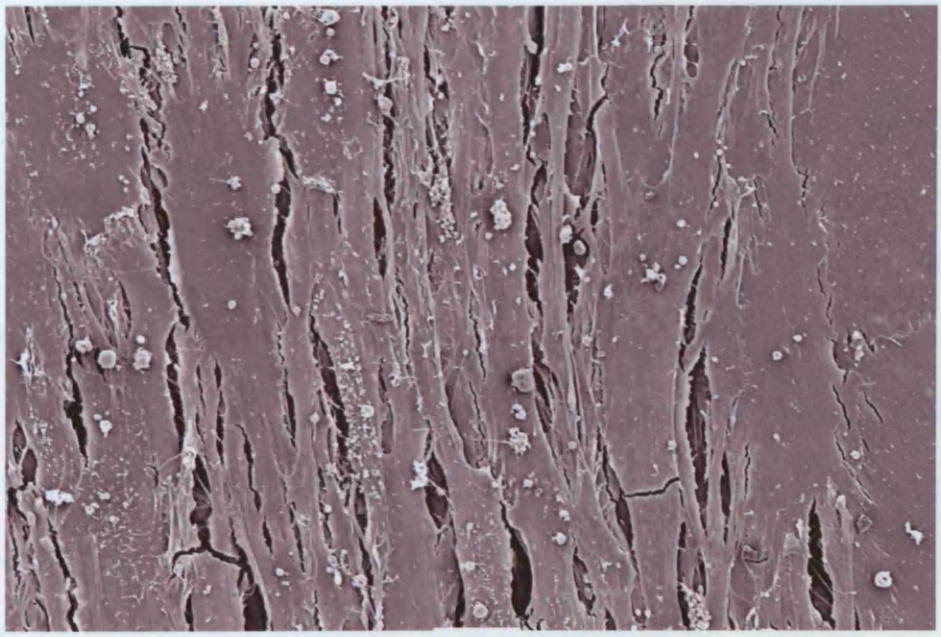
b

Day14. Both control (a) and Colloss (b) treated groups have reached confluence. Cells on Colloss treated titanium (b) appear to have a more random orientation compared to the control group.

Figs.8a and b. BMSSCs. Day 21. S.E.M. magnification X 600.



a



b

Fig.8. Cells at day 21 showing confluence of cells grown on both control (a), and Colloss (b) pre-treated titanium. Cell morphology is similar in both groups.

4.5. Discussion

A prerequisite for the stability and longevity of uncemented orthopaedic implants is periprosthetic bone formation and implant osseointegration. The osseointegration of orthopaedic implants relies on the formation of bone directly onto prosthetic surfaces. In the case of uncemented femoral hip replacement stems implanted within the femoral canal, there are bone marrow stromal stem cells in close proximity to the prosthesis which have the ability to differentiate into osteoblasts,which in turn can form bone. A greater understanding of the interactions between bone marrow stromal stem cells and orthopaedic biomaterials may facilitate the development of techniques to enhance periprosthetic bone formation and osseointegration. Previous studies have examined the effects of different biomaterials on the growth and differentiation of bone marrow stromal stem cells. Ohgushi et al 1996 [169] examined the behaviour of rat marrow stromal stem cells on bioactive glass ceramics with and without an apatite layer. It was shown that osteoblastic differentiation was enhanced by the presence of the apatite layer as evidenced by increased levels of Alkaline Phosphatase, mineral deposition and bone specific GLA protein. [169]

Other studies have examined the growth of mature osteblasts on titanium and the effects of surface treatments on cell behaviour. Ching-Hsin Ku et al, 2002, demonstrated that aging titanium alloy to reduce metal ion release had the result of increasing significantly the proliferation of osteoblasts.[168] However, few studies have examined the growth and differentiation characteristics of multipotential bone marrow stromal stem cells cultured on titanium alloy. In this chapter we have demonstrated the ability of bone marrow stromal stem cells to attach and proliferate on titanium alloy, a material in common use for the manufacture of hip replacement femoral components. The ability of Colloss treated titanium to enhance the osteoblastic differentiation of

bone marrow stromal stem cells is supported by the increased levels of Alkaline Phosphatase at day 7 and the increased osteopontin levels at days 14 and 21. Levels of Alkaline Phosphatase and osteopontin were several magnitudes greater than control samples. In control cultures ALP expression remained low and did not change significantly between days 7, 14 and 21. In contrast, the experimental groups showed a peak in ALP expression at day 7, which was followed by a sharp fall in ALP activity to day 14.

Osteopontin is a glycosylated phosphoprotein with a molecular weight of 32,600 which is expressed by osteoblasts and may be involved in cellular attachment. It binds to calcium and interacts with the CD44 cell surface glycoprotein which can attach to collagen type I, fibronectin and hyaluronic acid.[170,171] There is evidence that it is involved in the process of osteoclastosis and remodelling.[143,172] During the cascade of osteogenesis, peak bone formation only usually occurs after Alkaline Phosphatase expression in the latter stages of osteogenesis.[118] In control cultures, Ostepontin expression was minimal and did not vary significantly during the study period. In the Colloss groups, osteopontin expression was three times that of the control cultures on day 14 and more than 10 times the mean day 21control value. This would suggest that Colloss is stimulating the osteogenic differentiation of bone marrow stromal stem cells on the titanium surfaces. Surprisingly, there was no difference in the rate of type I collagen synthesis between control and Colloss cultures.

Evidence of a stimulatory effect of Colloss on the osteoblastic differentiation of BMSSCs comes from increased early Alkaline Phosphatase expression and raised osteopontin levels. It is possible that when medium was initially added to the titanium disks, Colloss may have been washed off the titanium surface. This would result in a 1:40 dilution of Colloss suspension within the medium. On subsequent exchange for fresh medium, Colloss would be removed from culture. This may explain why

enhancement of Alkaline Phosphatase activity was confined to the early culture period. However, procollagen expression was not significantly influenced in the Colloss pre-treated titanium samples and morphological appearances were non- specific.

The effect of bone morphogenic protein on the proliferation and differentiation of bone marrow stromal stem cells grown on titanium has been investigated previously. Van Den Dolder et al cultured rat bone marrow stromal cells on machined titanium with and without the addition of BMP2. Unlike this study, BMP 2 was added to culture medium.[173] In contrast to our results, BMP 2 did not have an effect on cell proliferation, however, the time points used were 4, 8 and 16 days which differed from this study. BMP 2 appeared to induce the osteogenic differentiation of stromal stem cells resulting in the early expression of Alkaline Phosphatase, calcium deposition and matrix formation.

Summary

The results of this study provide evidence in support of the osteogenic potential of Colloss to stimulate the differentiation of bone marrow stromal stem cells along osteoblastic lineage pathways. Evidence, however, is inconclusive because collagen type 1, a marker of osteogenesis was not elevated in the Colloss treated titanium samples.

5.Chapter 3

Proliferation and Differentiation of Bone Marrow Stromal Stem Cells on Hydroxyapatite Granules Treated with Colloss

5.1. Introduction

Hydroxyapatite (Ca_{10} $(PO4)_6$ $(OH)_2$) has a composition similar to that of bone. Its biocompatible and bioactive properties have favoured its use as a bone graft substitute material and as a coating to enhance osseointegration on implants. [174, 175, 176, 155] Its bioactivity is characterised by its ability to ellicit a biological reaction at its surface which results in chemical bonding to bone.[176]

When hydroxyapatite (HA) is exposed to tissue fluids, a hydroxylcarbonate apatite layer forms which resembles the mineral phase of bone and provides a bioactive interface for new bone formation, resulting in bonding with bone.[177, 178, 179, 180] When bonding is established, collagen fibres may interdigitate with the hydroxyapatite crystals near the interface. Such chemical bonding is strong and the interface shear strength may exceed the cohesive strength of hydroxyapatite to attached prosthesis.

It has been established that Hydroxyapatite is osteoconductive, hence supports the proliferation, migration and differentiation of osteoprogenitors, but there remains controversy as to whether it is has any osteoinductive properties.[26, 59, 60, 176]

The general consensus is that hydroxyapatite is not greatly osteoinductive, but hydroxyapatite implanted in extraskeletal sites in animals and humans has been shown to support new bone formation.[181, 182, 183] Some authors consider this evidence of osteoinductive capacity, but others attribute this apparent osteoinduction to binding of endogenous bone morphogenic proteins onto the hydroxyapatite surface.[181, 182, 184]

When applied to the surface of orthopaedic implants, such as femoral stems of total hip replacements, hydroxyapatite facilitates bone ingrowth onto implant surfaces. [156] The close apposition of bone to prosthetic joints is desirable to enhance implant stability, reduce the risk of loosening and improve implant longevity.

There is an abundance of osteoblasts within the bone marrow which can produce bone matrix onto the hydroxyapatite surface. In addition, multipotential bone marrow stromal stem cells within the medullary cavity provide a potential source of osteoprogenitors to facilitate new bone formation during the process of implant osseointegration.

The excellent osteoconductivity of HA confers an ability to support the growth, proliferation and migration of osteoprogenitors and osteoblasts during the process of osseointegration.[27,59,185] Hydroxyapatite's low osteoinductive capacity could be overcome by combining it with an osteoinductive substance to form a composite biomaterial. HA can absorb liquids, hence can be used as a carrier for soluble factors. These properties make HA ideal as a carrier for Colloss suspension.

5.2. Aim and Hypothesis

Aim - The aim of this chapter is to examine and compare the growth and differentiation of human bone marrow stromal stem cells cultured on hydroxyapatite and hydroxyapatite pre-soaked with a Colloss suspension

Hypothesis – Colloss will enhance the osteogenic differentiation of bone marrow stromal stem cells cultured on the surface of hydroxyapatite.

5.3. Materials and methods

5.3.1. Bone marrow stromal stem cell harvest and culture.

Using methods described in chapter 1(3.3.1), human bone marrow stromal stem cells were harvested from the iliac crest of an adult female volunteer undergoing an elective orthopaedic procedure. Bone marrow stromal stem cells were isolated from marrow using a Ficoll gradient technique, seeded onto culture flasks and incubated in monolayer, at 37°C in 95% air and 5% carbon dioxide at 100% humidity. The standard medium used for culture comprised Dulbecco's modified Eagles medium (4500mg/L glucose/L, L-glutamine and sodium pyruvate, Sigma Aldrich D6429), with 1% penicillin / streptomycin. (Sigma-Aldrich Co Ltd, Irvine UK) and 10% bovine foetal calf serum (FCS).

5.3.2. Hydroxyapatite preparation and seeding with BMSSCs

Granules of coarse pore foam Hydroxyapatite, particle size 4-8mm were used in this study (Plasma Biotal Limited Whitecross Road Industrial Estate, Tideswell, SK17 8PY). Four granules of hydroxyapatite were placed in individual wells of a 24 well culture plate.

In order to minimise variations in hydroxyapatite surface area between control and Colloss cultures, granules were visually inspected and those which appeared identical in size were selected. Such inspection is subjective and is unlikely to eliminate differences in surface area, but the aim was to reduce such variation to a minimum. To further standardise the surface area of Hydroxyapatite the same number of granules were placed in each culture well.

Pre-treatment of granules with Colloss was performed by pipetting Colloss suspension (Colloss dispersion, concentration 1.4d.m./ml, Ossacur AG, Oberstenfeld, Germany) to cover the surface of hydroxyapatite granules. Granules were then left to stand at room temperature for 1 hour. Granules were then transferred to another 24 well plate to exclude Colloss which may have spread onto the base of the wells. The control cultures comprised Hydroxyapatite granules alone without Colloss pre-treatment.

Bone marrow stromal stem cells were seeded onto the hydroxyapatite granules by pipetting medium containing bone marrow stromal stem cells at a density of 800,000 cells/ml onto the surface of hydroxyapatite granules. After seeding, the granules were incubated for 30 minutes to allow the cells to attach to the HA prior to adding 2mls of standard medium to each well. Cell proliferation, total DNA, Osteopontin and procollagen were measured in triplicate cultures. After 24 hours the granules were transferred to a new 24 well culture plate to exclude bone marrow stromal stem cells which may have been present on the bottom of the culture wells.

5.3.3. Cell proliferation assay using alamar™ blue Assay

Cell proliferation was measured using an alamar blue assay, utilising techniques described in chapter 1 (3.3.5.) on days 7, 14 and 21 of culture.

5.3.4. Total DNA using Hoescht 33258

Total DNA was measured on days 7, 14 and 21 using the fluorometric DNA binding dye, Hoescht 33258 as described in chapter 1 (3.3.6.).

5.3.5. Alkaline Phosphatase assay on Cobas Bio analyser

Alkaline Phosphatase was measured on days 7, 14 and 21 using a technique adapted from Oreffo et al 1998 [119] which is described in chapter 1(3.3.7.).

5.3.6. Type I Procollagen radio immunoassay

Type 1 collagen synthesis was measured on days 7, 14 and 21 of culture with a type I procollagen radioimmunoassay (Orion Diagnostica, Orion Corporation. PO Box 83, 02101 Espoo, Finland) using the techniques described in chapter 1(3.3.10.).

5.3.7. Osteopontin immunoassay

Osteopontin was measured on days 14 and 21 using a human osteopontin enzyme immunometric assay kit (TiterZyme ® EIA. Assay Designs, Inc. 800 Technology Drive Ann Arbour, MI 48108 U.S.A). The technique has previously been described in chapter 1(3.3.9.).

5.3.8.Scanning Electron Microscopy

Samples of stem cell - Hydroxyapatite composites were prepared for electron microscopy after selected periods of culture. The methods of sample processing is described in chapter 2 (4.3.9). Microscopy was performed using a JEOL model JSM-550LV Scanning Electron Microscope.

5.3.9. Statistics

On the basis of the Kolmogorov – Smirnov statistic it was determined that variable data was non parametric. Statistical analysis of variables was therefore performed using the Mann Whitney U-test.

5.4. RESULTS

5.4.1. Cell proliferation.

There was no significant increase in cell proliferation rate between control and Colloss groups on days 1, 3, 9 and 14. On day 21 control cultures had a higher proliferation rate than Colloss cultures (p=0.004). In both groups cell proliferation increased significantly from days 1 to 3 (p=0.004). From days 3 to 9 cell proliferation in the Colloss group decreased significantly (p=0.016), whereas cell proliferation rate in control cultures did not change significantly. From days 9 to 14 cell proliferation fell significantly and increased significantly between days 14 and 21 (p=0.004). In both groups proliferation rate peaked on day 21 (Fig.1.).

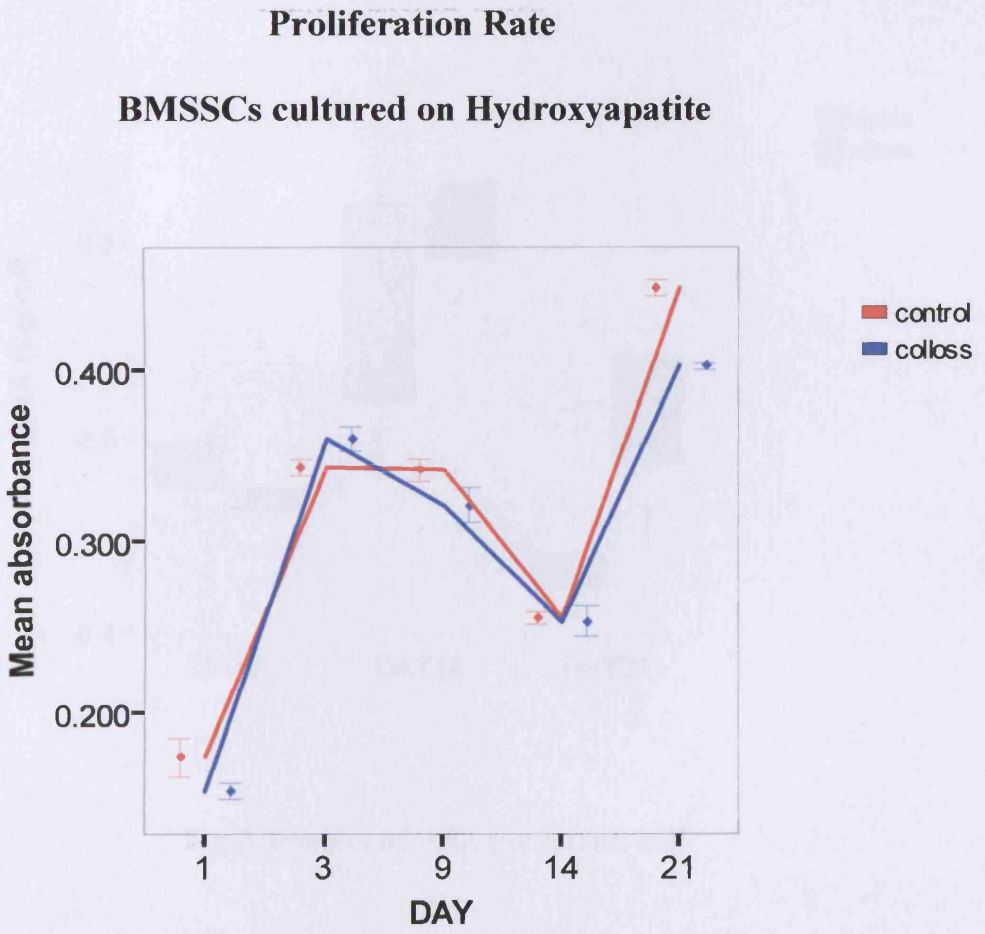


Fig.1. Cell proliferation using alamar blue. Mean absorbance per well
Error bars show mean +/- 1 standard error

5.4.2. DNA

On days 7 and 14 there was no difference in DNA between control and Colloss groups. On day 21 DNA was significantly higher in Colloss than control cultures. In control cultures there was no significant difference in DNA between days 7 and 14. DNA decreased significantly between days 14 and 21 (p=0.05) and days 7 and 21 (p=0.05). In the Colloss group DNA increased significantly from days 7 to 14 (p=0.050) and decreased significantly from days 14 to 21 (p=0.050, Fig.2).

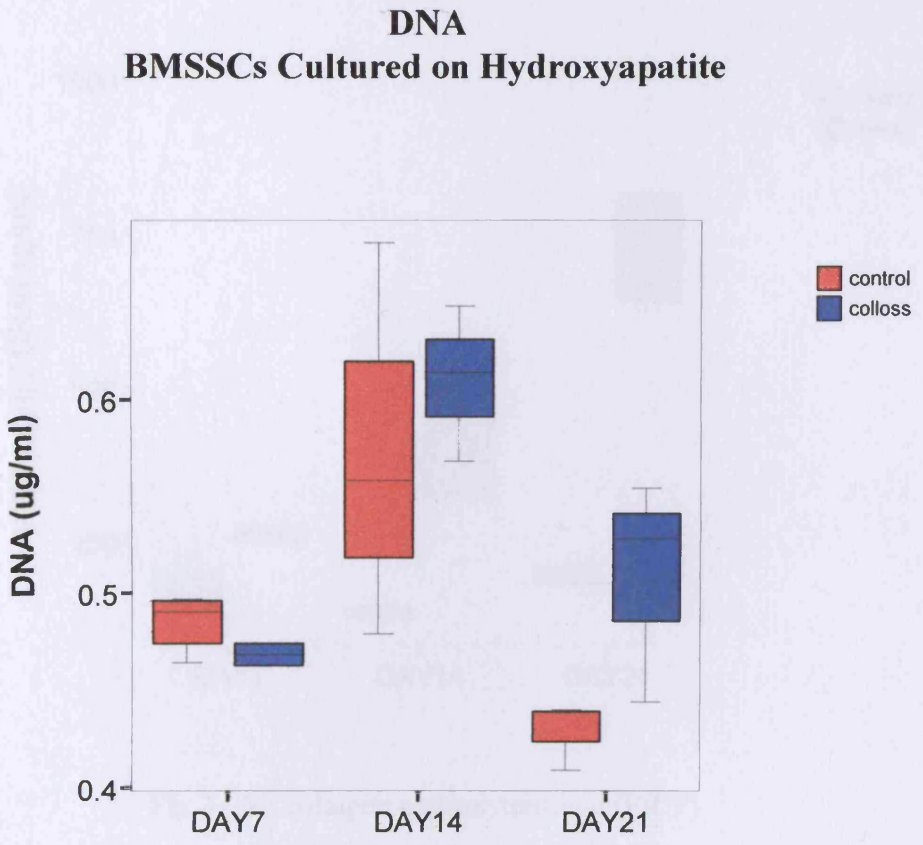


Fig.2. Boxplot of DNA per culture well

5.4.3. COLLAGEN TYPE I SYNTHESIS

Procollagen expression on days 7, 14 and 21 was significantly greater on HA pre treated with Colloss (p=0.050). In both control and Colloss groups procollagen levels peaked on day 21. There was a significant increase in procollagen levels from days 7 to 14 and 14 to 21 in the Colloss group (p=0.050, Fig.3.).

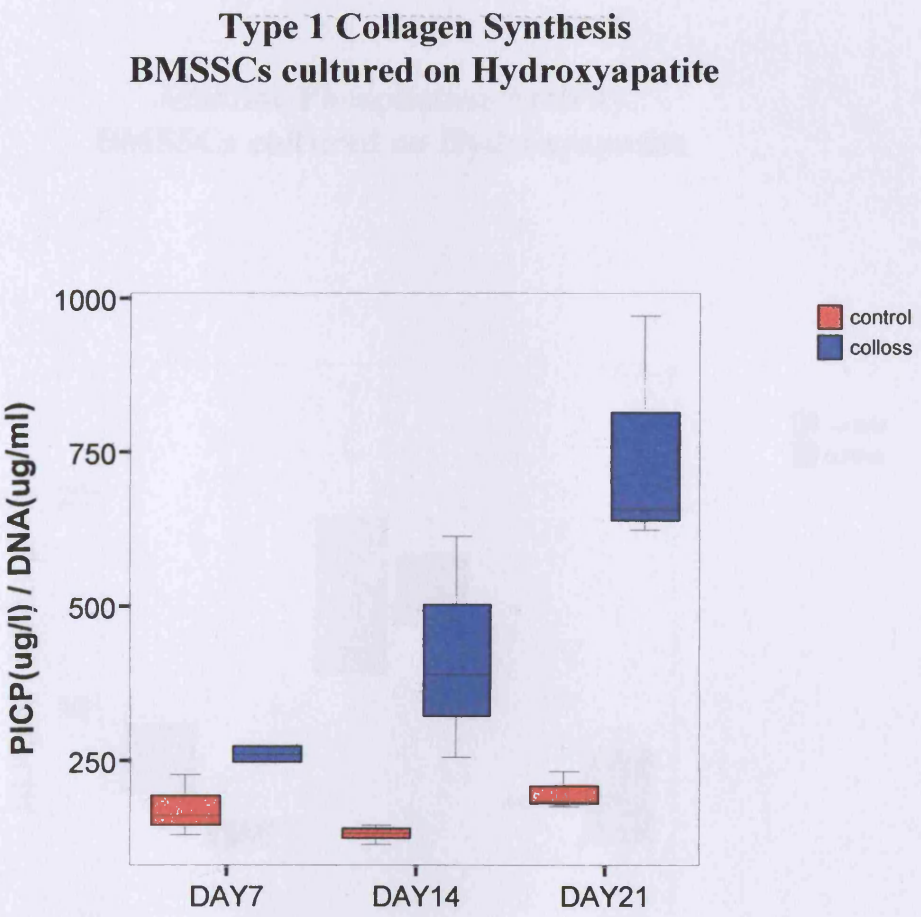


Fig.3. Pro collagen carboxyterminal (PICP) Radioimmunoassay for Collagen Type 1 synthesis. Boxplot of PICP per well standardised for DNA.

5.4.4 Alkaline Phosphatase Activity

Alkaline Phosphatase activity was significantly higher in control than Colloss cultures on day 7 (p=0.05). However, there was no difference between groups on days 14 and 21. ALP expression increased significantly from day 7 to day 14 to reach peak levels in Colloss but not control cultures (p=0.05). In both groups Alkaline Phosphatase decreased significantly from days 14 to 21 (p=0.05).

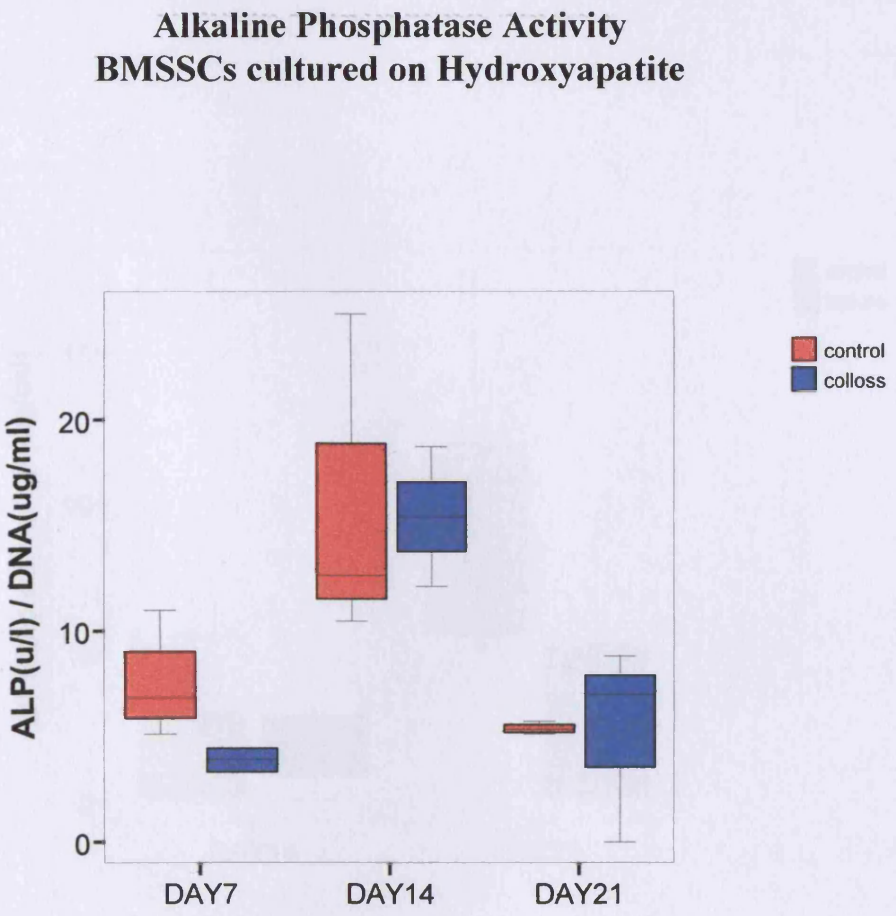


Fig.4. Alkaline phosphatase with Cobas Bio.
Boxplot of Alkaline phosphatase activity per well standardised for DNA.

5.4.5. Osteopontin

There was no significant difference in osteopontin expression between Colloss and control groups on days 14 and 21. The increase in osteopontin expression between days 14 and 21 was not significant in Colloss (p=1.000) or control (p=0.127) groups. (Fig.5.).

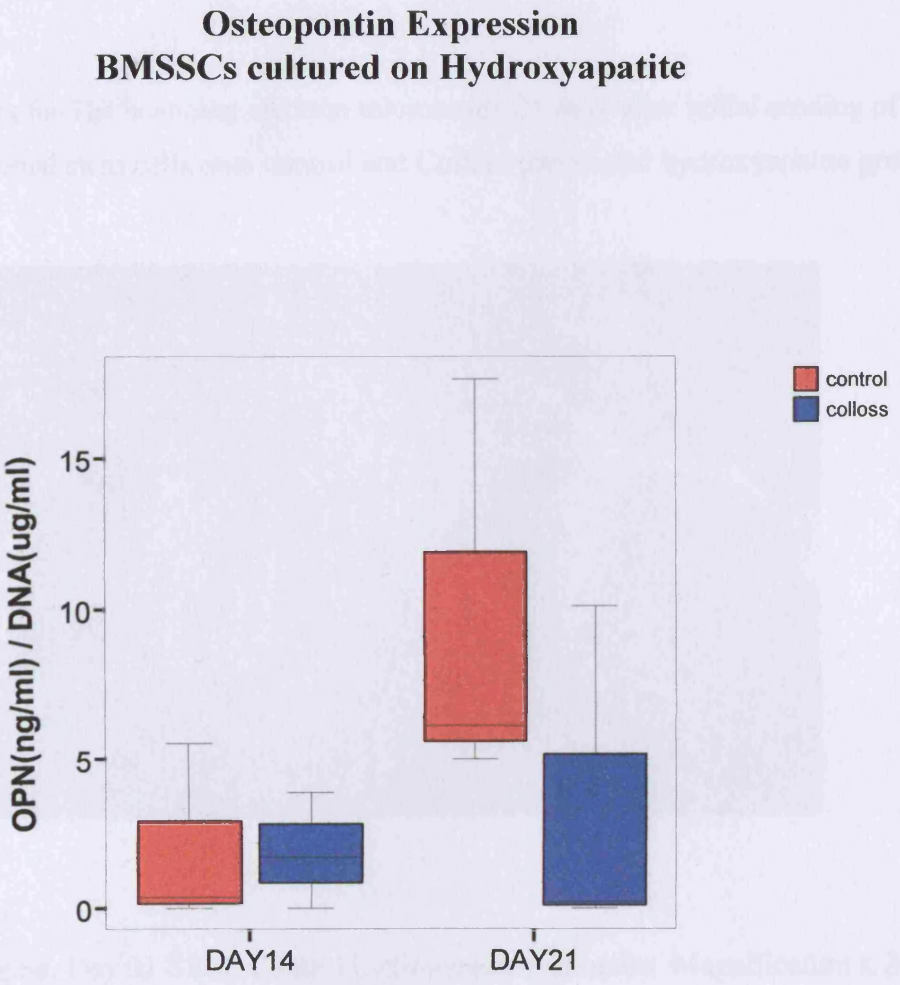


Fig.5. Boxplot of Osteopontin per culture well, standardised for DNA.

5.4.6. Cell morphology

On day 21 the control group sample cells were confluent, but maintained an elongated, spindle shaped fibroblastic appearance. (Fig.6a and 7a). In contrast, cells cultured on the Colloss treated HA granules did not have a spindle shaped morphology but were broader, with a polygonal appearance. (Fig.6b and 7b)

Figs 6a-7b: Scanning electron microscopy 21 days after initial seeding of bone marrow stromal stem cells onto control and Colloss pre-treated hydroxyapatite granules.

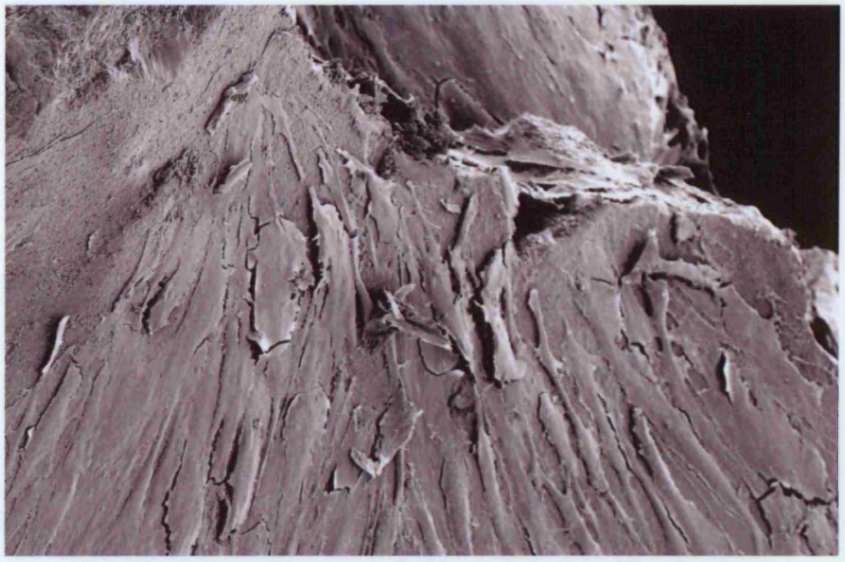


Fig.6a. Day 21 SEM. Control hydroxyapatite granules. Magnification x 200.

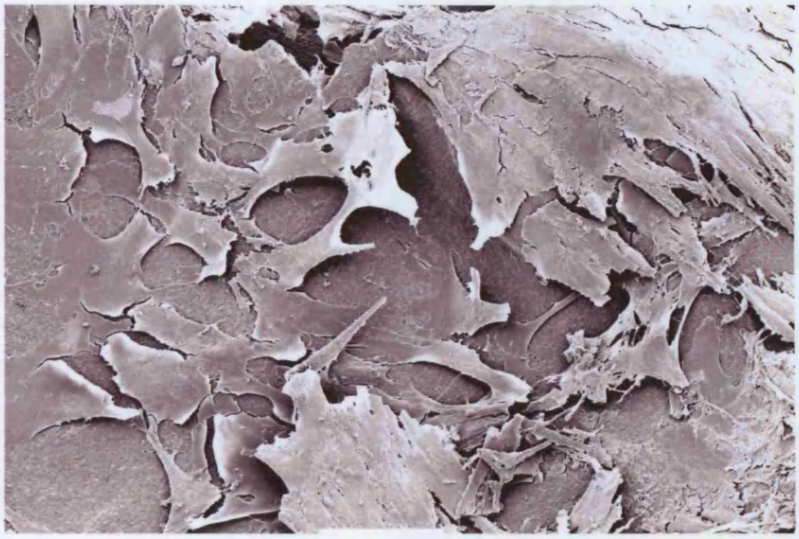


Fig.6b. Day 21. S.E.M. Colloss treated hydroxyapatite granules. Magnification x 200.

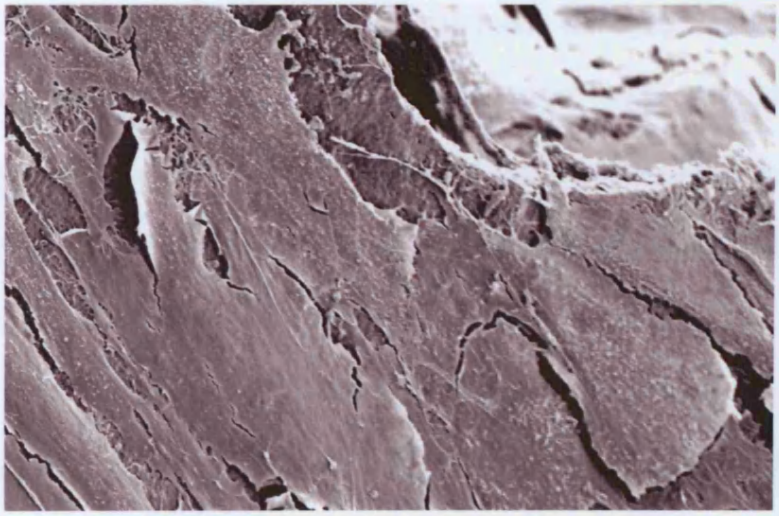


Fig.7a. Day 21.S.E.M. Control group.
Magnification X 600.

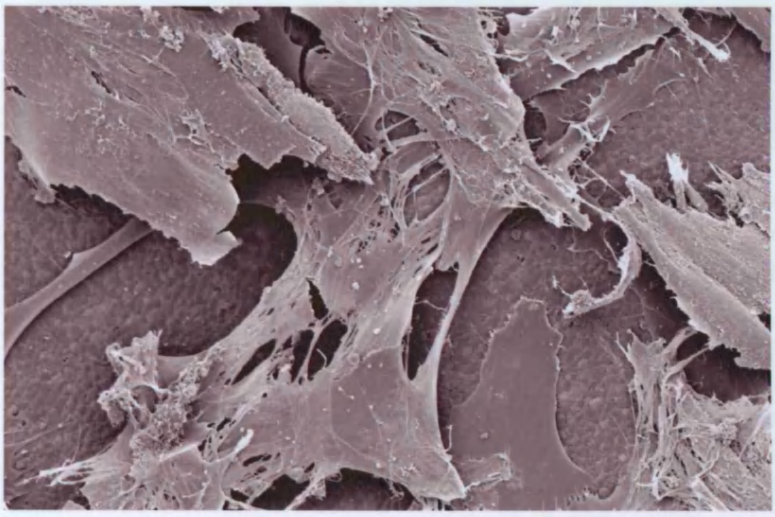


Fig. 7b. Day 21 SEM. Colloss treated hydroxyapatite.
Magnification X 600.

5.5. Discussion

The pattern of cell proliferation in both control and Colloss treated HA samples was similar for the culture duration with peaks at days 3 and 21. Proliferation rates were very similar, with no difference detected until day 21, when control culture proliferation rates were marginally but significantly higher as measured by alamar blue, which by measuring the metabolic activity of cell cultures, provides an indirect measure of cellular proliferation.

Total DNA levels did not differ between groups until day 21 when total DNA was greater in the Colloss group. Assuming that the amount of DNA per cell is constant, this would suggest a greater number of cells in the Colloss cultures on day 21. Previous *in vitro* studies have demonstrated that HA supports the osteogenic differentiation of bone marrow stromal stem cells. Ohgushi et al, 1993, implanted HA pre-loaded with autologous bone marrow and a control group of HA without bone marrow into subcutaneous sites in rats.[186] It was found that pre-loaded HA supported the formation of bone and cells expressed bone GLA protein (BGP) mRNA, whereas in non-loaded

control hydroxyapatite implants significant bone formation was not seen and BGP mRNA was not detected.

Hydroxyapatite has been shown to enhance the osteoblastic differentiation of bone marrow stromal stem cells cultured in the presence of osteogenic supplements. Nordstrom et al, 1999, described the culture of rat marrow cells on hydroxyapatite in the presence of osteogenic supplements.[26] Cells grown on HA differentiated to form mineralised nodules positive for ALP stains, but cells grown on control culture dishes were less intensely stained and showed enzyme activity half that of cells grown on HA.

In previous studies, methods of enhancing the osteogenic differentiation of bone marrow stromal cells cultured on hydroxyapatite have been investigated. Kim et al cultured bone marrow stromal stem cells on HA with and without the addition of recombinant Human Bone morphogenic protein 2. Alkaline Phosphatase levels were not enhanced by BMP 2, suggesting that's its ability to stimulate the differentiation of stromal stem cells into osteoblasts is limited. Furthermore, osteocalcin, a later marker of osteogenesis was not detected throughout the duration of the study period.[187]

In this study, unlike that of Ohgushi et al 1993[186] , osteogenic supplements were not added to cultures to initiate osteoblastic differentiation. We found that Colloss treated HA stromal cell cultures showed a peak ALP expression on day 14, however levels were no greater than those in control cultures. Osteopontin, a marker of osteogenic differentiation usually expressed later than ALP, did not change significantly between days 14 and 21 and did not differ in level between the control and Colloss treated samples. There was, however, a stepwise increase in procollagen from days 7 to 21, levels of which were significantly higher in the Colloss pre-treated HA granules.

The results of this study do not provide conclusive evidence that Colloss can enhance the osteoblastic differentiation of stromal stem cells cultures on hydroxyapatite granules. The process of osteoblastic differentiation and the formation of osteoid can be

divided into three stages: proliferation, matrix maturation and mineralisation. Alkaline Phosphatase is expressed early during this process with collagen and osteopontin expression occurring later during matrix maturation and mineralisation. Although Alkaline Phosphatase was expressed by cells cultured on HA pre-treated with Colloss, we did not observe increased levels of Alkaline Phosphatase in the Colloss group compared to control cultures, as would be expected during the early stages of culture to signify osteoblastic differentiation. Surprisingly Alkaline Phosphatase levels were significantly higher in the control group at day 7. Also, Colloss pre-treated hydroxyapatite did not enhance the expression of osteopontin, a marker of osteoblastic differentiation detected in the latter stages of differentiation. Despite this, a finding consistent with the enhancement of extracellular matrix formation and osteoblastic differentiation were the levels of collagen type I we observed which increased during the culture period to reach levels on average three times those of the control cultures. Collagen type I is a component of bone matrix and its presence suggests a positive effect of Colloss on the osteoblastic differentiation of bone marrow stromal stem cells. Although Collagen type I can also be produced by fibroblasts, the morphological features of bone marrow stromal stems cultured for 21 days suggested an osteoblastic phenotype, lending further support for the osteoinductive capacity of Colloss.

Summary

Hydroxyapatite pre-treated with Colloss suspension supported the growth of bone marrow stromal stem cells. Evidence of the ability of Colloss to enhance osteoblastic differentiation was, however, inconclusive as only one of the osteogenic markers, type 1 collagen synthesis was raised in the Colloss pre-treated hydroxyapatite cultures.

6. Chapter 4

The Effect of Colloss on the Osseointegration of Titanium

6.1. Introduction

The stability of femoral stem prostheses relies on the stable fixation between the implant and host bone. Osseointegration is the process by which host bone grows onto implant surfaces to provide stable support. The osseointegration of titanium femoral prostheses is important to provide implant stability, limit loosening and improve the longevity of total hip replacements. The ability of implants to osseointegrate with host bone may be compromised by the presence of bone defects adjacent to the implant at the time of surgery. This is more often a problem during revision surgery when bone defects may be extensive. In many centres current practice involves packing of bone defects with allograft. Unfortunately there are disadvantages associated with the use of allograft and therefore research has been undertaken to find alternatives.

6.2. Aim and Hypothesis

Aim - The aim of this part of my study is to stimulate bone formation within bone gaps adjacent to shotblasted titanium implants to enhance osseointegration using Colloss.

Hypothesis - Colloss will enhance bone formation within bone defects adjacent to porous titanium implants and promote their osseointegration in a sheep tibia gap model.

6.3. Materials and Methods

Course for intending licensees under the Animals (Scientific Procedures) Act 1986

As a prerequisite for personal license application the author attended a course for intending licensees under the Animals (Scientific Procedures) Act 1986.

Personal and project licenses under the animals (Scientific Procedures) Act 1996

A personal license was then obtained after application to the Home Office.

All animal experiments in this study were covered by the existing departmental project licence.

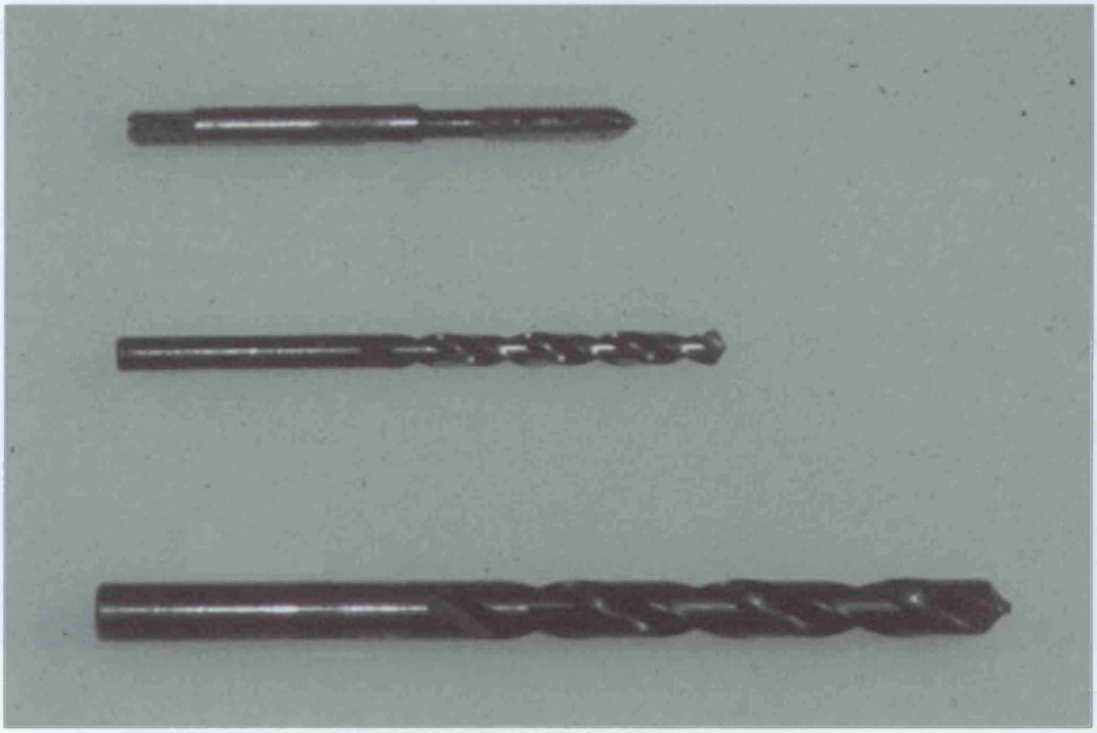
6.3.1. Tibia Plug Gap Model

This model was devised to provide a means of inserting cylindrical shotblasted titanium implants into the shafts of sheep tibia to create circumferential gaps of 1 and 2mm between the implant and cortical bone.

A system was devised to allow accurate concentric placement of cylindrical titanium implants into the tibia. Drills were used to make holes in the tibia for placement of titanium implants (Fig.1.) To create a 1mm gap between the implant and cortex, a 7mm hole was created in the medial cortex using a 7mm diameter drill. A bush of external

diameter 6.9mm, internal diameter 4.4mm was then placed in the 7mm hole, with the distal end resting in the medullary canal on the intact lateral cortical bone. The outer borders lying flush against the medial cortical wall. A 4.3mm diameter drill passed through the center of the bush with which it lay flush, to drill the lateral cortex.

Fig1. Drills and Tap from top to bottom, Tap, 4.3mm diameter drill and 7mm diameter drill



This bush was then removed and replaced with a second bush with the same 6.9mm external diameter, but an internal diameter of 5.1mm. After tapping the drill hole, a cylindrical shot-blasted titanium implant of diameter 5mm, threaded distally, was placed in the center of the bush and screwed firmly into the lateral cortex.

To create a 2mm circumferential gap between implant and cortex, a titanium implant with a diameter of 3mm in the non-threaded region was used.

Fig.2. Shotblasted titanium implants

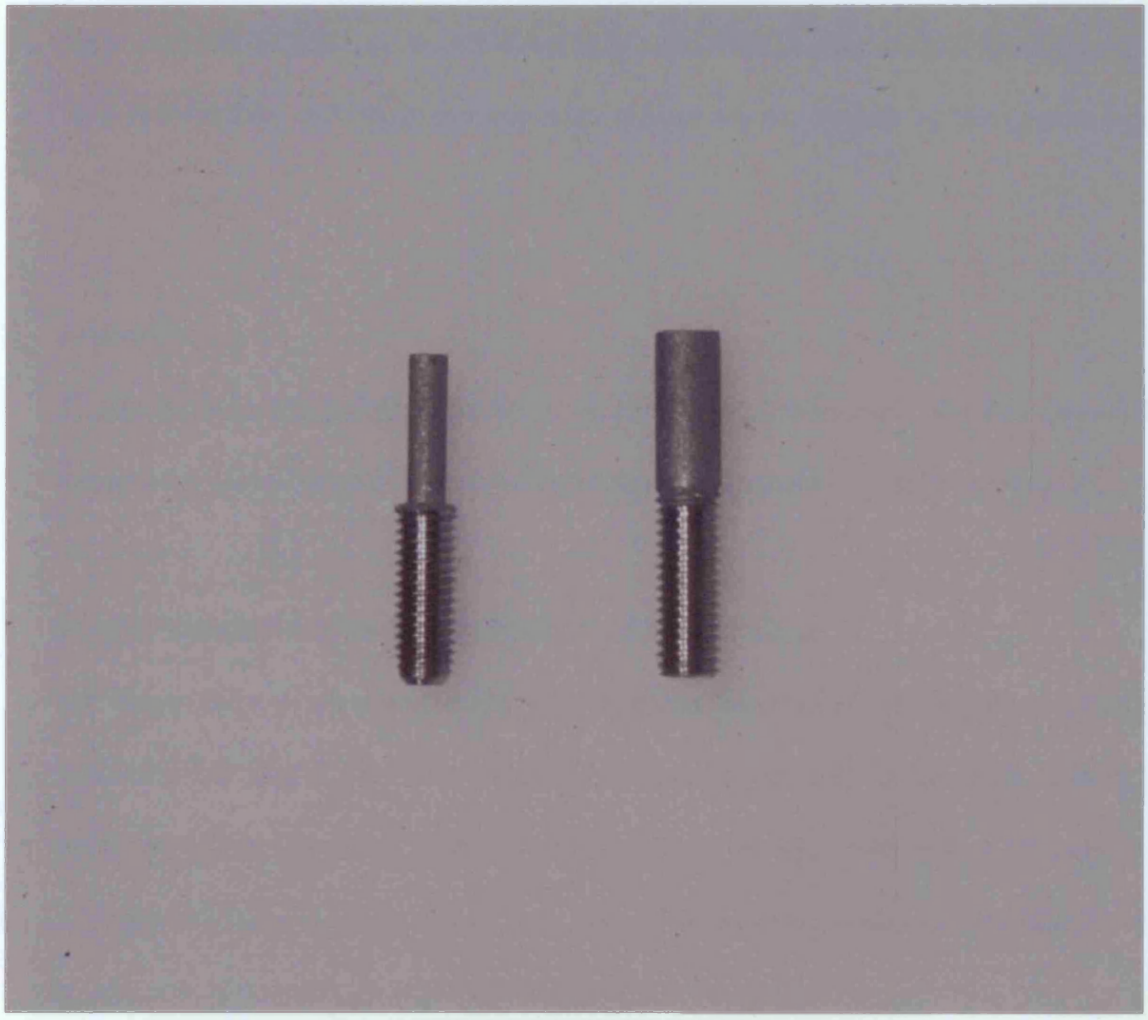


Fig.2. Implant (A) left, 3mm diameter and implant (B), right, (5mm diameter).

All implants were made from titanium 318 (Ti Al6 V4, Titanium International Ltd. Keys House, Birmingham B33 OSP, UK). Implants were engineered to the specified shape at the Centre for Biomedical Engineering, Stanmore Middlesex. Implant B was used to create 1mm periprosthetic gaps. The implant is 25mm in length and 5mm

diameter, threaded in the distal 12mm. A slot was engineered on the proximal circular surface to allow engagement of a screwdriver for ease of implant insertion.

Implant A is 25mm in length. The proximal 13mm of the implant is 3mm in diameter. The distal 12mm is threaded to engage the lateral cortex and is of diameter 5mm. As with implant A, there is a slot in the proximal circular surface for engagement of a screwdriver. All implants were shot-blasted on the surface of the non-threaded region.

Animals

12 female mule breed sheep aged between 2 and 4 years were used. Prior to operation sheep were group housed and following surgery sheep were housed in single pens.

6.3.2. Pre-medication and Induction of anaesthesia

All sheep were given pre-medication comprising Rompun 0.4ml (9.328mg) xylazine hydrochloride (Bayer plc Animal Health Business Group, Bury St Edmunds, Suffolk) by intramuscular injection. Induction of anaesthesia was achieved by the administration of ketamine hydrochloride 180ml and 3mg of midazolam intravenously. Sheep were intubated and anaesthesia was maintained with 2% halothane.

6.3.3. Operative Procedure

Sheep were positioned on the right lateral side. The skin of the tibia was shaved and prepared with iodine. Drapes were applied to isolate the operative field (Fig.3). Antibiotic prophylaxis was administered in the form of 100mg ceftiofur (Pfizer Ltd, Walton Oaks, Surrey, UK) by intramuscular injection (2mls solution).

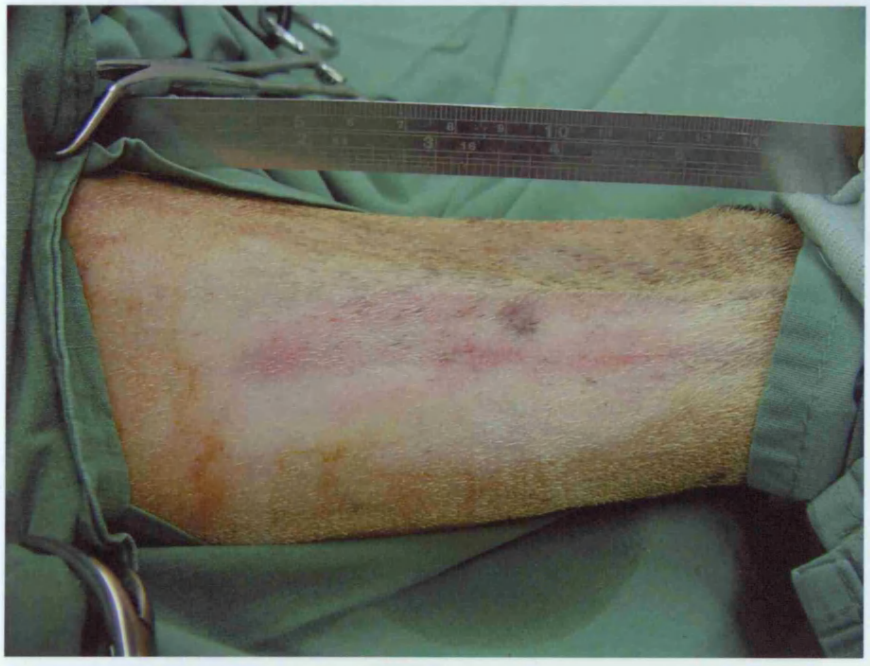
A longitudinal incision was made along the medial subcutaneous border of the tibia, starting approximately 3 cm below the tibial tuberosity, extending distally through skin

and fascia. A self retaining retractor was used to displace the skin and fascia laterally and expose the periosteum. A longitudinal incision was made in the periosteum, which was then elevated using a periosteal elevator.

From proximal to distal, four 7.1 mm diameter holes were drilled through the medial cortex of the tibia. The first hole was placed approximately 3cm distal to the tibial tuberosity. The remaining 3 holes were placed distally to the first one, with a distance of 25mm between the centers of each hole. A 4.3 mm hole was then drilled in the lateral cortex concentric to the first hole in the medial cortex using a bush as a guide (Fig.5). The drill hole in the lateral cortex was then tapped, again using a bush as a guide.

Fig 3. Preparation of skin and draping prior to operation

medial aspect of sheep tibia



Copious irrigation of the drill holes with saline was performed to remove bone and marrow debris. The operative field was then dried using gauze swabs. Titanium implants were inserted into the medial drill hole and screwed into the lateral cortex

using a screwdriver. Two of each type of implant was inserted to create two 1mm gaps and two 2mm gaps (Fig.6).

Fig. 4. Medial aspect of sheep tibia with four 7mm diameter drill holes.

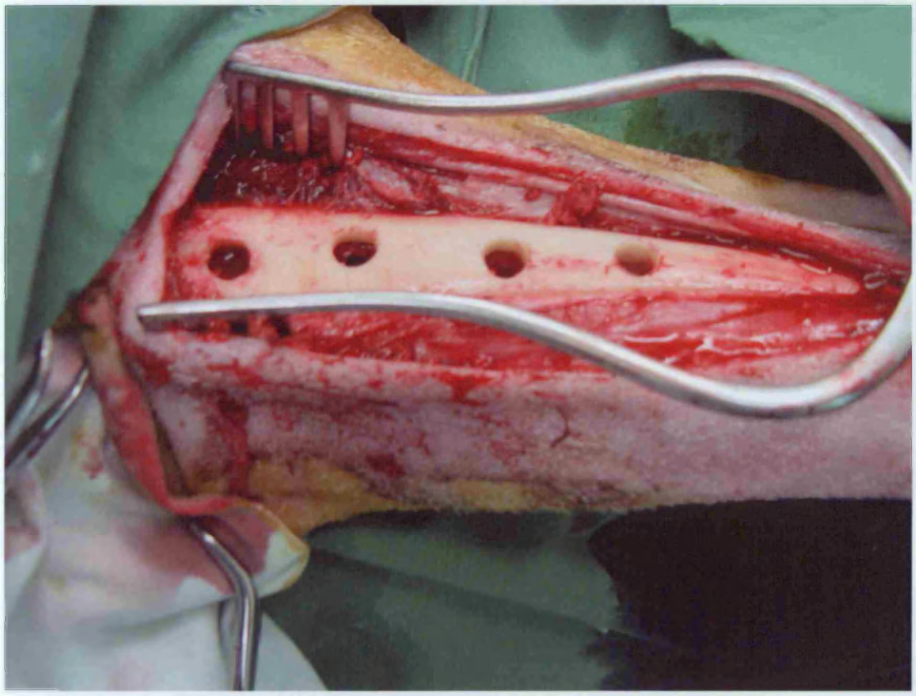
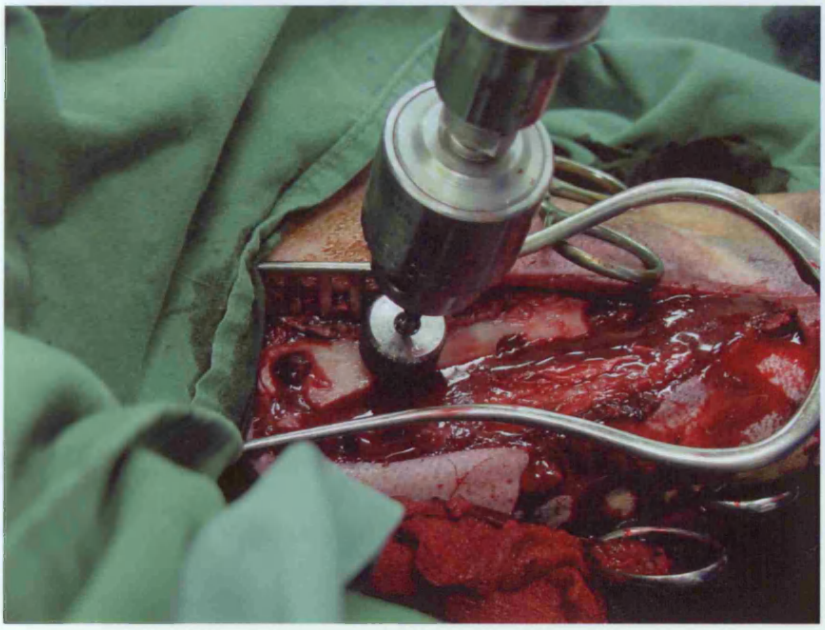


Fig. 5. Drilling of 4.3 mm holes in lateral cortex using bush

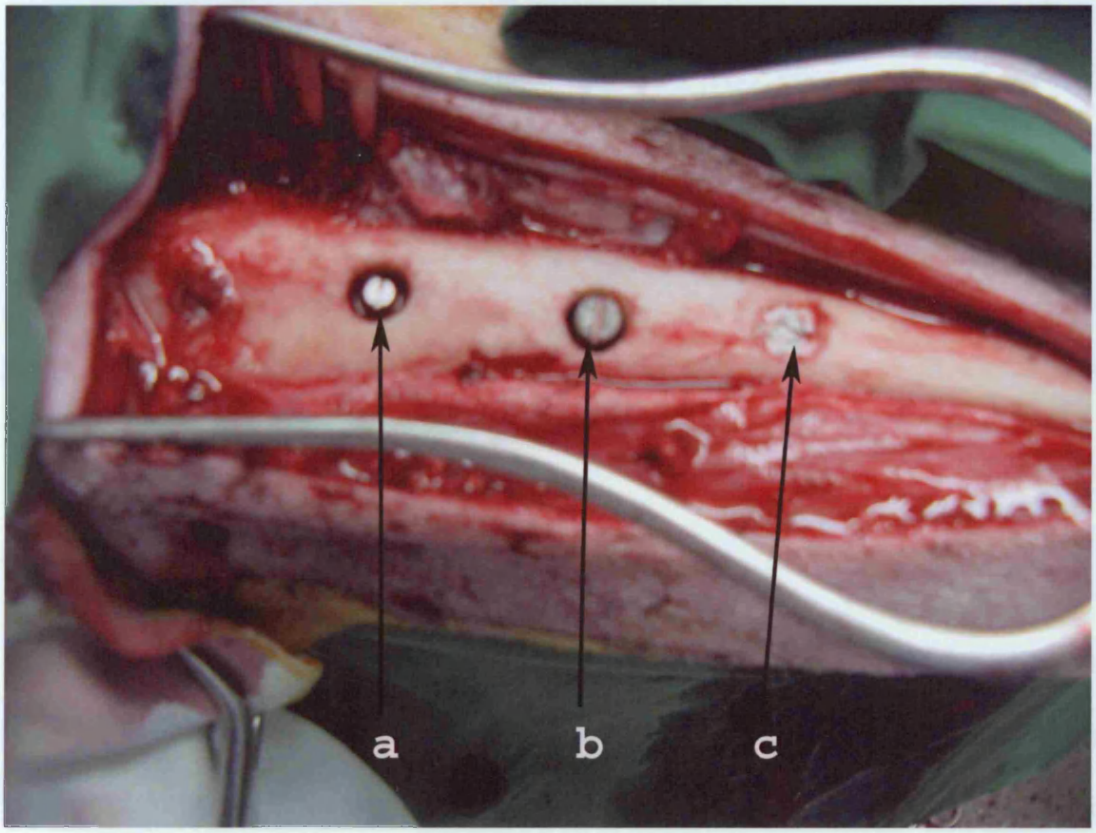


Collagen lyophilisate was inserted into one of the 1mm gaps and one of the 2mm gaps between the implant and cortical bone. The remaining two periprosthetic defects were left empty and used as controls.

The subcutaneous tissues were approximated with continuous 2-0 vicryl. Skin was closed with interrupted 2-0 vicryl sutures. Opsite wound dressing (Smith & Nephew, Cambridge, UK) was sprayed onto the wound, which was covered with dry gauze which was then secured with a bandage and TED compressive stocking.

Six procedures were performed for each time period of 4 and 8 weeks. The position of the pins and their treatments (+/- Colloss) was varied in a rotatory fashion for each of the 6 operations. (Two combinations were therefore repeated).

Fig 6. Titanium pins in situ.



3mm (a) and 5mm (b) diameter titanium pins. Colloss placed into gap (c).

6.3.4. Post operative care

After surgery, animals were housed in single pens. The antibiotic ceftiofur 100mg was administered intramuscularly once a day for 5 days post-operative days.

6.3.5. Harvest of specimens

At 4 and 8 weeks following surgery, sheep were sacrificed by intravenous injection with 60mls of phenobarbitone. The tibia were harvested, radiographed and then processed for histology.

6.3.6. DEXA Scanning

Bone mineral density (BMD) of tissue within the gap between the titanium implants and cortical bone was measured by DEXA using an x-ray bone densometer (Hologic model QDR-1000/W, Massachusetts, USA) in the anterior- posterior plane.

2mm peri-prosthetic defects

Bone mineral density was measured in a selected area 0.25 mm from the titanium implant for a width of 1mm.

1mm peri-prosthetic defects

BMD was measured in a selected area 0.25mm from the titanium implant for a width of 0.5mm. BMD of the cortical bone immediately superior and inferior to the periprosthetic gap was measured, averaged and used as the control value. The BMD of the peri prosthetic defect was expressed as a ratio of this control value. In both the 1 and 2mm defects the above process of selection was performed 6 times for each implant and averaged to give a single value.

6.3.7. Histology

Specimens were fixed in 10% formal saline for 2 weeks and then processed in serial changes of alcohol (10%, 30%, 30% , 70%, 90% and 100%) for durations of 1,3,1,1,1, and 3 days respectively. Following dehydration in alcohol, specimens were placed in chloroform to remove fat from specimens. Four chloroform changes were made over a 72 hour period. Specimens were then placed in 100% alcohol for a further 4 days. Alcohol was changed 3 times during this period.

Specimens were placed in a solution of alcohol 50% / Acrylic resin 50% (LR White Resin, London Resin Company, Reading, Berkshire, England) for 72 hours, then acrylic resin 100% for 1week under vacuum. Resin was polymerised by the addition of LR white accelerator LR White Resin, London Resin Company, Reading, Berkshire, England).

Preparation of slides

Specimens within resin were cut in the coronal plane along the length of the implants using an EXAKT saw. Specimens were glued onto Perspex slides and ground between 60-90 um using and EXACT grinding machine before being polished. Slides were stained using 1% Toludine Blue in 1% Borax for 15 minutes, followed by Paragon for a further 15 minutes. Slides were then left to dry for 24 hours before application of coverslips.

6.3.8. Scanning electron microscopy. (S.E.M)

Specimens which had previously been embedded in resin were sectioned through the implant in the coronal plane. The specimens were then mounted onto S.E.M. stubs and sputter coated with Palladium for 2 minutes to provide a coating of approximately 15nm. (Emitech K550, Emitech Ltd, South Stour Ave, Ashford, Kent, TN23 7RS). S.E.M was performed using a JEOL scanning electron microscope. (JSM-550LV).

S.E.M Images were saved as BMP (Bitmap) images and then converted to JPEG (Joint Picture Experts Group) images. Images were merged using Adobe Photoshop version 7 software (Adobe Systems Incorporated, USA) to provide a complete image of each implant and adjacent cortical bone.

Using Ziess^{TM} image analysis software, the area of the gap between bone and titanium implant was measured to a depth of 3mm from the cortical bone surface. The presence or absence of bony apposition to the implant surface was also noted.

6.3.9. Statistics

The Kolmogorov – Smirnov test was used to determine the normality of data. On the basis of the Kolmogorov – Smirnov statistic, it was determined that variable data was non-parametric, hence comparisons of bone mineral density and pin gap area were compared using the Mann Whitney U test.

6.4. Results

6.4.1. Bone mineral density within periprosthetic defects

6.4.1.1. *Four Week Specimens*

4 week group 1mm periprosthetic defects.

Average BMD ratio in the 1mm periprosthetic defects was 0.7142 compared to 0.6644 in the 1mm defects pre-filled with Colloss. This difference was not significant (p=0.485).

4 week group 2mm periprosthetic defects.

Average BMD ratio in the 2mm periprosthetic defects was 0.6431 compared to 0.7621 in the 2mm defects pre-filled with Colloss. This difference was not significant (p=0.310).

6.4.1.2. *Eight Week Specimens*

8week group 1mm periprosthetic defects.

Average BMD ratio was 0.8154 in the 1mm defects pre-filled with Colloss, compared to 0.7561 in the control group. This difference was not significant (p=0.394).

8 week group 2mm periprosthetic defects.

Average BMD ratio was 0.7305 g/cm^2 in the 2mm defects pre-filled with Colloss, compared to 0.6420 g/cm^2 in the control group. This difference was not significant (p=0.240).

4 weeks vs 8 weeks; 2mm defects.

There was no significant difference in BMD ratio between the 4 and 8 week time periods for 2mm Colloss (p=1.0) or the 2mm control group (p=0.873).

4 weeks vs 8 weeks; 1mm defects

There was no significant difference in BMD ratio between the 4 and 8 week time periods for 1mm Colloss (p=0.150) or the 1mm control group (p=0.423).

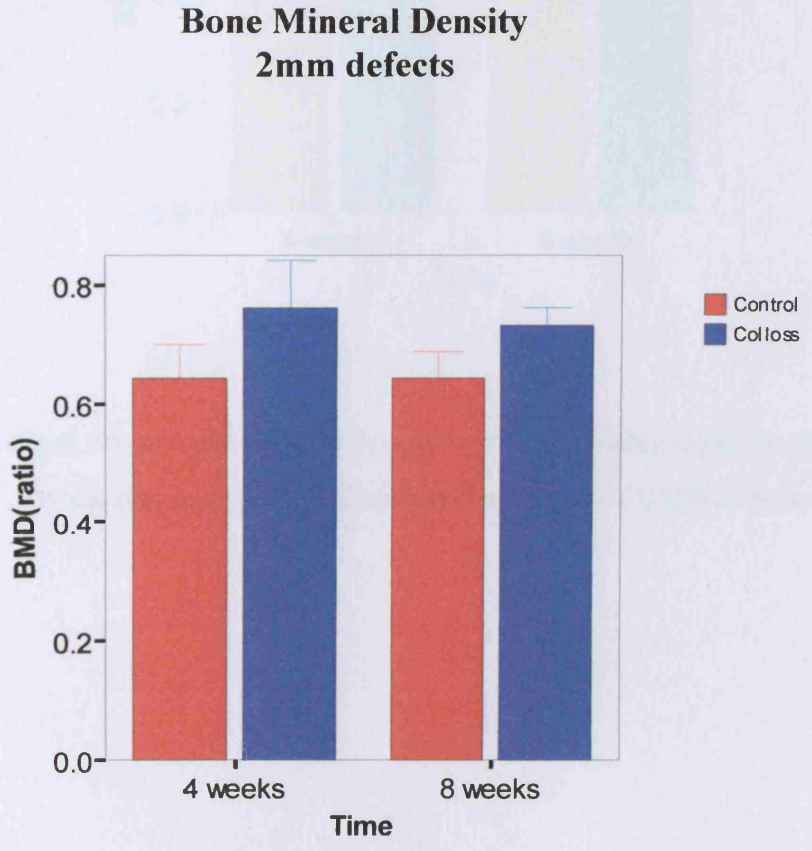


Fig.7. Mean bone mineral density within 2mm periprosthetic defects. Four and eight week specimens. BMD Expressed as a ratio of BMD of bone adjacent to defect. Error bars represent +/- 1 standard error of the mean

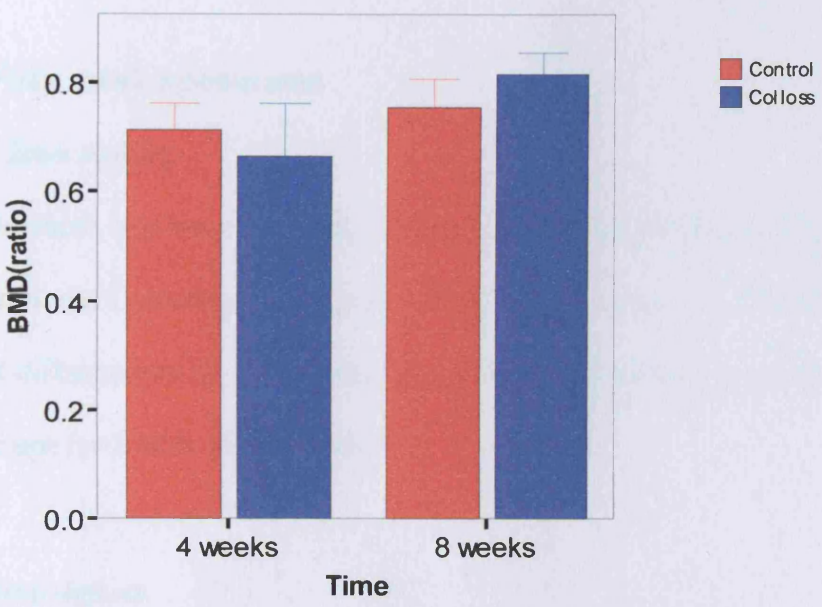


Fig.8. Mean bone mineral density within 1mm periprosthetic defects. Four and eight week specimens. BMD Expressed as a ratio of BMD of bone adjacent to defect.

6.4.2. Bone formation within Periprosthetic defects

The area of periprosthetic defects at four and eight weeks is shown in fig.9.

6.4.2.1. Four week specimens

1mm and 2mm defects.

At 4 weeks there was no significant difference in the periprosthetic gap area between 1mm control and Colloss groups. (p=0.522) (Figs.10a, b and 11). There was also no significant difference in the periprosthetic gap area between the 2mm Colloss and 2mm control groups (p=1.000) (figs 12 and 13).

1mm vs 2mm defects.

At 4 weeks there was no significant difference in the periprosthetic gap area between the 1mm Colloss and the 2 mm control groups (p=0.055).

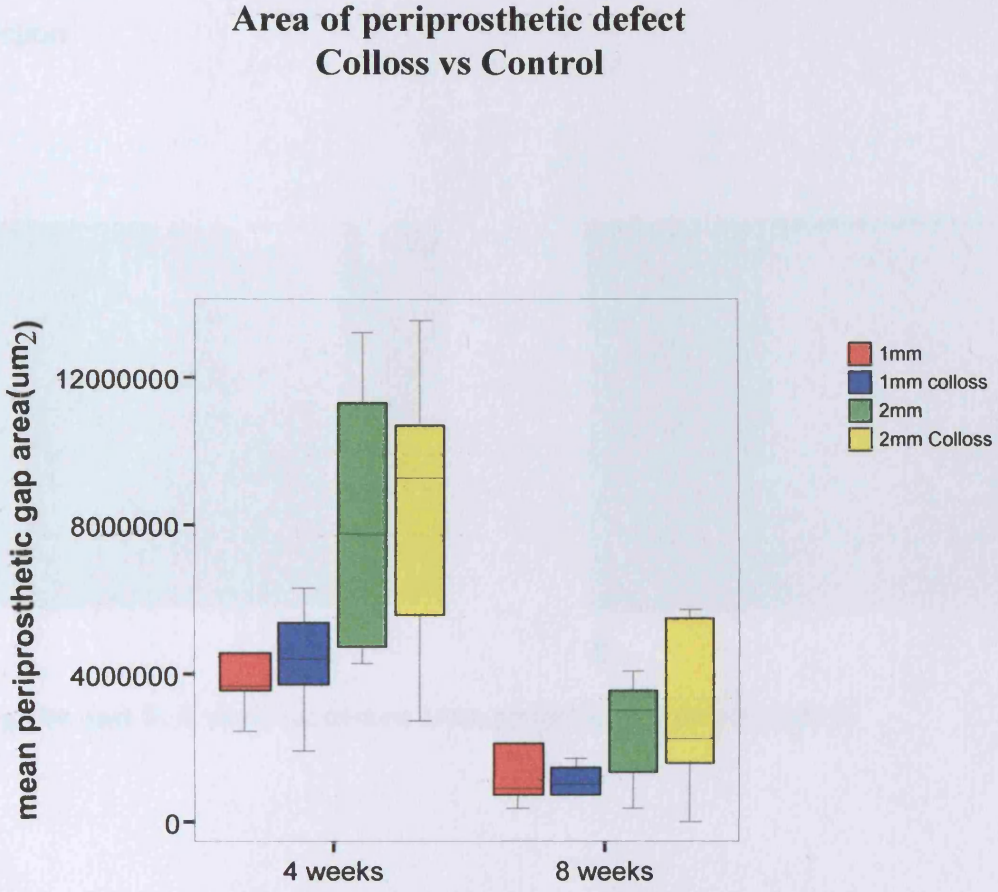
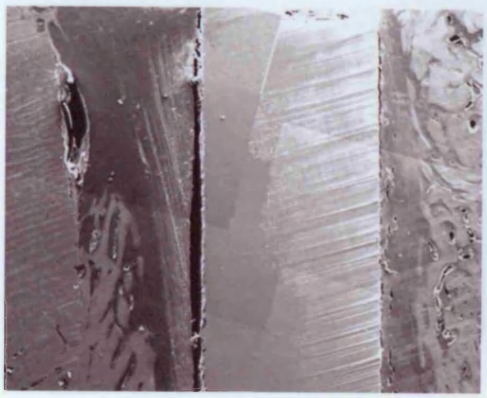


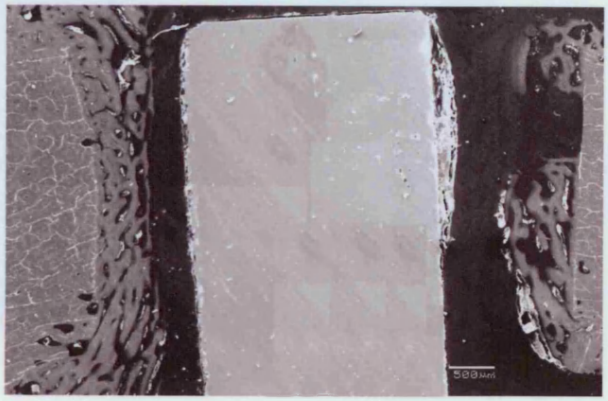
Fig.9. Boxplot of Periprosthetic defect area adjacent to titanium implants.

There was no significant difference in the periprosthetic gap between the 1mm and 2mm Colloss specimens (p=0.055). At 4 weeks the 1mm control group had significantly lower periprosthetic gap areas than the 2mm control group (p=0.025). There was no difference in the 2mm Colloss group and the 1mm control group at 4 weeks.

Figures 10-17. Scanning electron microscopy of Titanium implants in coronal section.



a



b

Fig.10a and b. 4 week specimens 1mm periprosthetic defect control

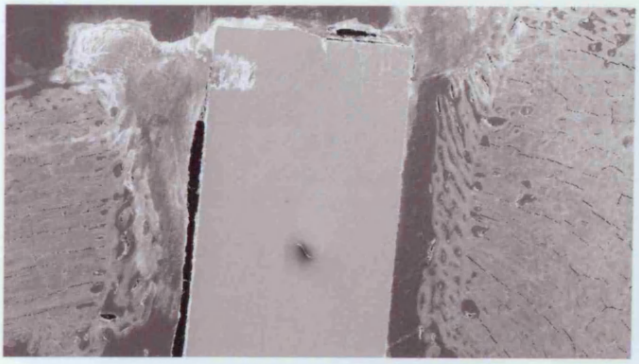
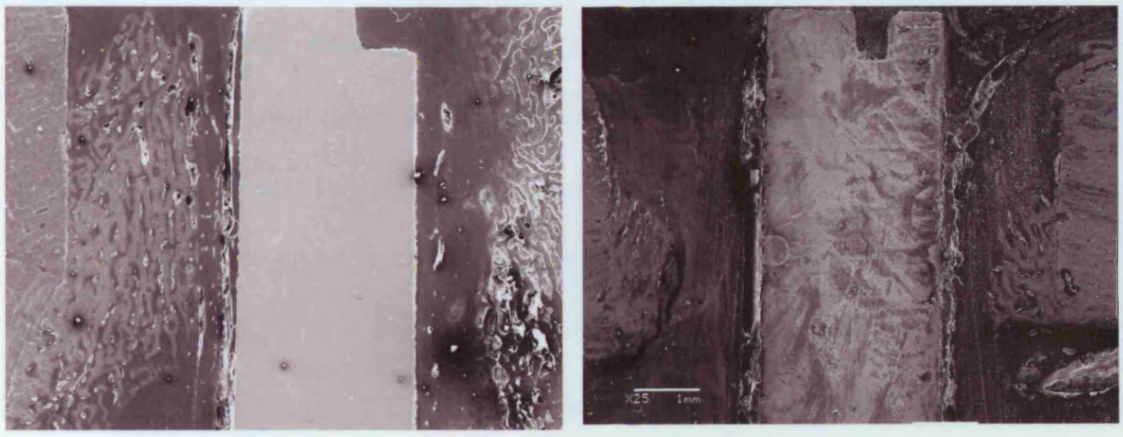
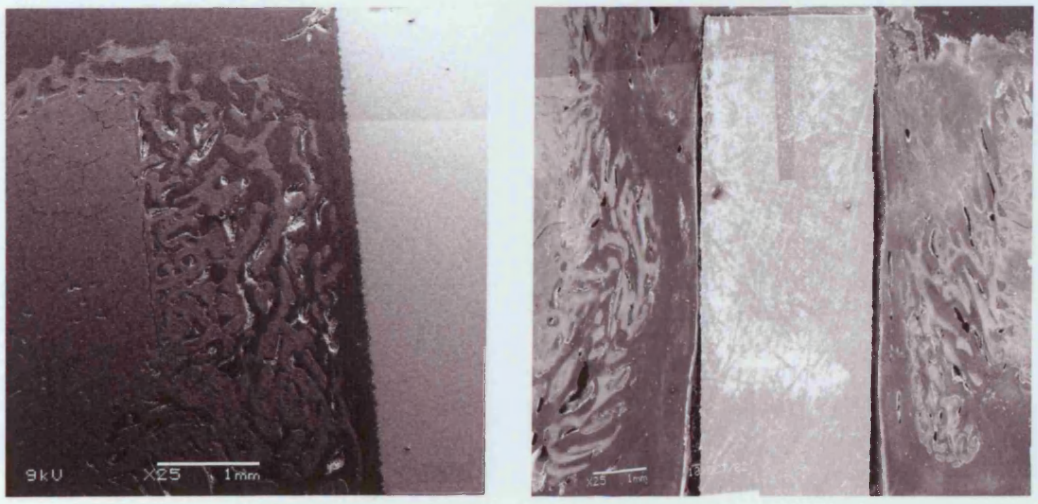


Fig. 11. 4 week specimen. 1mm defect with Colloss



a b

Fig.12a and b. 4 weeks 2mm periprosthetic defect control



a b

Fig. 13a and b. 4 weeks. 2mm periprosthetic defect with Colloss

6.4.2.2. Eight week specimens

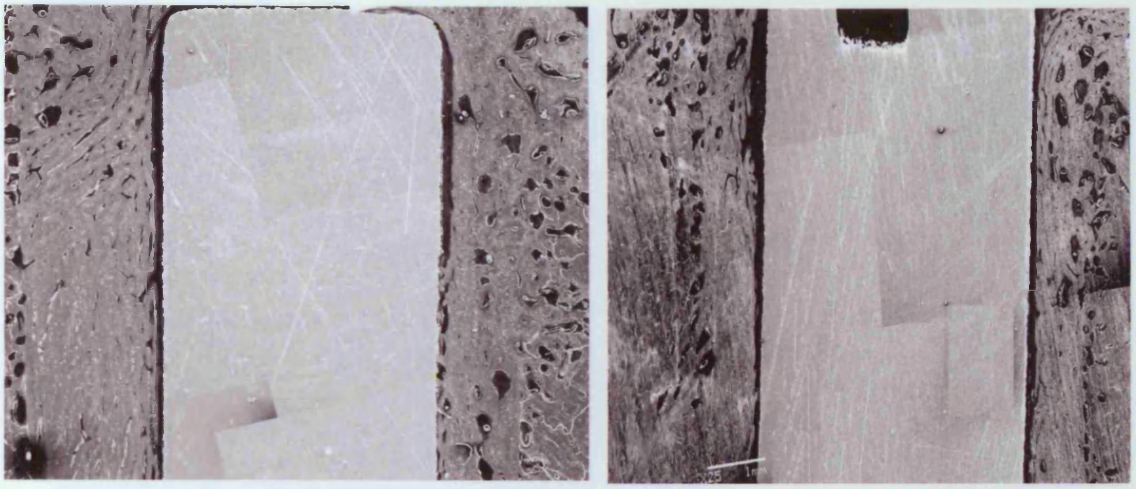
1mm and 2 mm defects.

At 8 weeks the peri implant gap was 2,548,695um^2 in the 1mm control group and 1,114,150um^2 in the 1mm Colloss group, but the difference was not significant (p=1.000, Figs 14 and 15). At 8 weeks there was no difference in peri-implant gap area between the 2mm control and Colloss specimens. (p=1.000, Figs 16 and 17).

1mm vs 2mm defects.

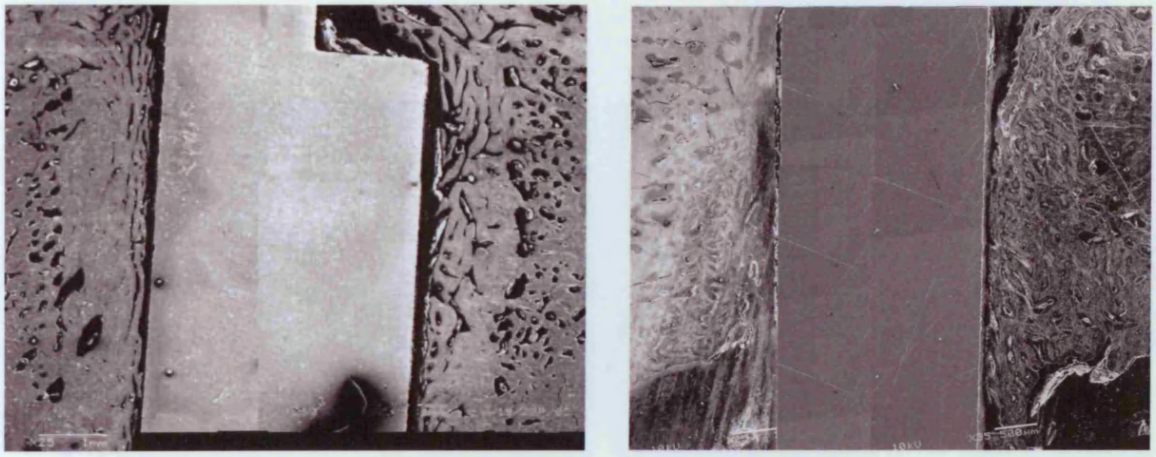
At 8 weeks there was no difference in peri-implant gap area between the 1 and 2 mm control groups (p=0.337). There was no difference in peri-implant gap area between the 2mm Colloss specimens and the 1mm control group gap area at 8 weeks (p=0.423).

There was no difference in peri-implant gap area between the 1mm Colloss group and the 2mm control group at 8 weeks (p=0.109). There was also no difference in peri-implant gap area between the 1mm Colloss and 2mm Colloss groups at 8 weeks (p=0.078).



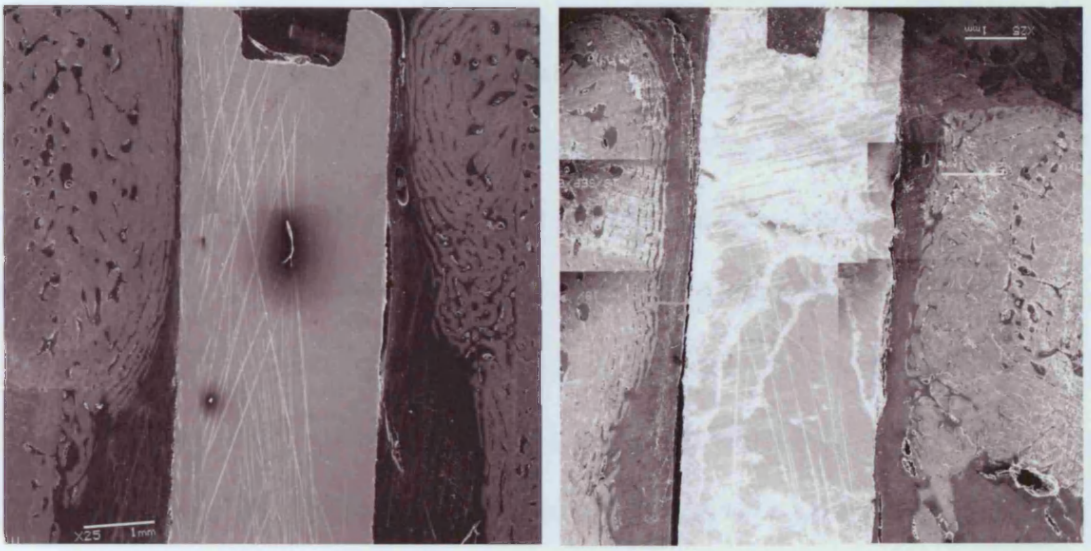
a b

Fig.14a and b. 8 week specimen. 1mm periprosthetic defect control



a b

Fig.15a and b. 8 week specimens. 1mm periprosthetic defect with Colloss



a b

Fig.16.a and b. 8 week specimens. 2mm periprosthetic defects control group

a b

Fig.17a and b. 8 week specimens. 2mm defects with Colloss

Note, in the Colloss containing specimen, (a). New bone formation with obliteration of gap on left. On the right is poorly mineralised new bone formation in close apposition to titanium implant.

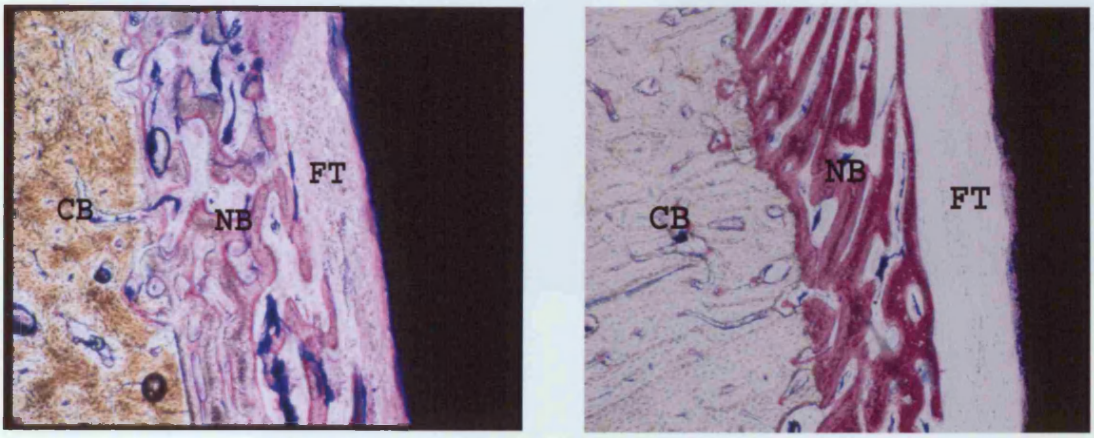
6.4.2.3. Four week vs eight week specimens

There was no difference in peri-implant gap area in the 1mm control groups between 4 and 8 weeks (p=0.055). However, there was a significant reduction in the peri-implant gap area between the 1mm Colloss 4 and 8 week specimens (p=0.004).

There was a significant reduction in peri-implant gap area in the 2mm control specimens between 4 and 8 weeks (p=0.004). There was also a significant reduction in peri-implant gap area in the 2mm Colloss specimens between 4 and 8 weeks (p=0.016).

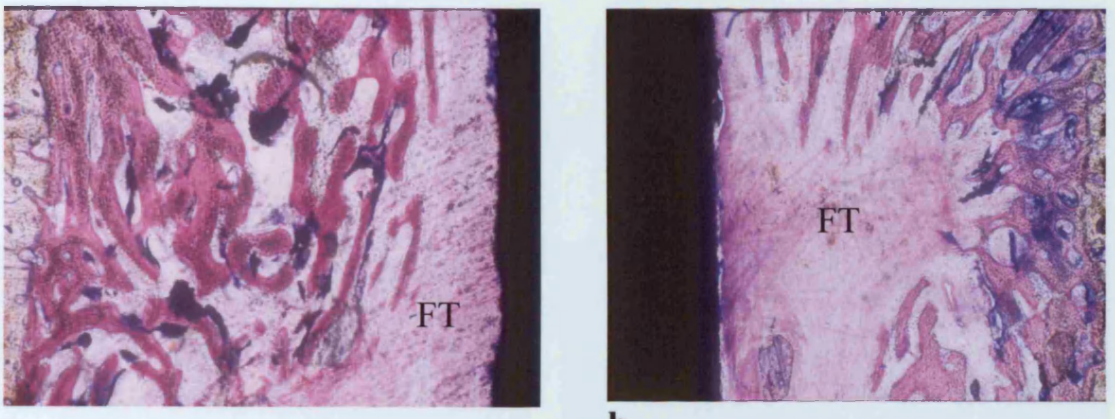
6.4.3. Descriptive Histology

6.4.3.1. Undecalcified Histology. 4 week Specimens



a b

Fig. 18a (control) and b(Colloss). Histology. 4 week specimen. 1mm periprosthetic defects. Stained with paragon and toluidine blue at the level of tibia cortex. There is immature woven bone formation in both the control and Colloss specimens. This lies in contact with the old mature lamellar bone(CB). In both groups the woven bone is separated from the titanium implant surface (Black region to right of picture) by interposition of fibrous tissue(FT)

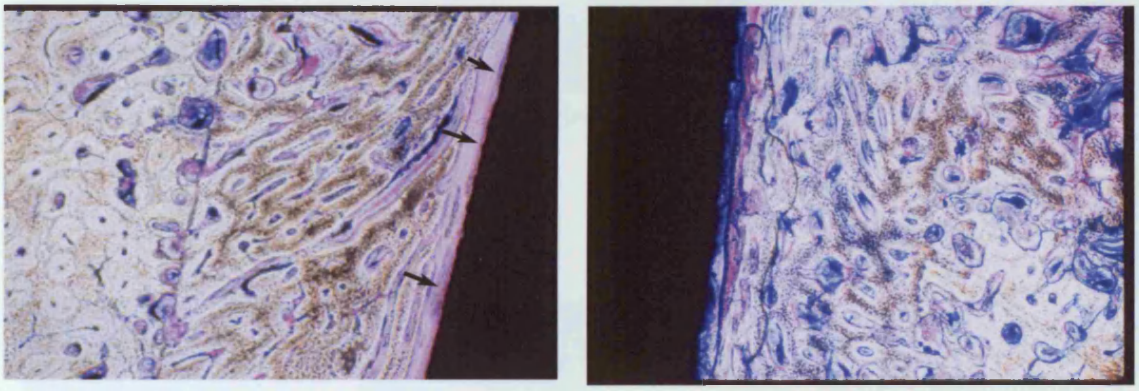


a b

Fig.19a(control) and b(Colloss). 4 week specimens with 2mm periprosthetic defects. Woven bone has formed in the control sample, in direct contact with old lamellar bone. Fibrous tissue is seen between the new bone and implant surface (FT).

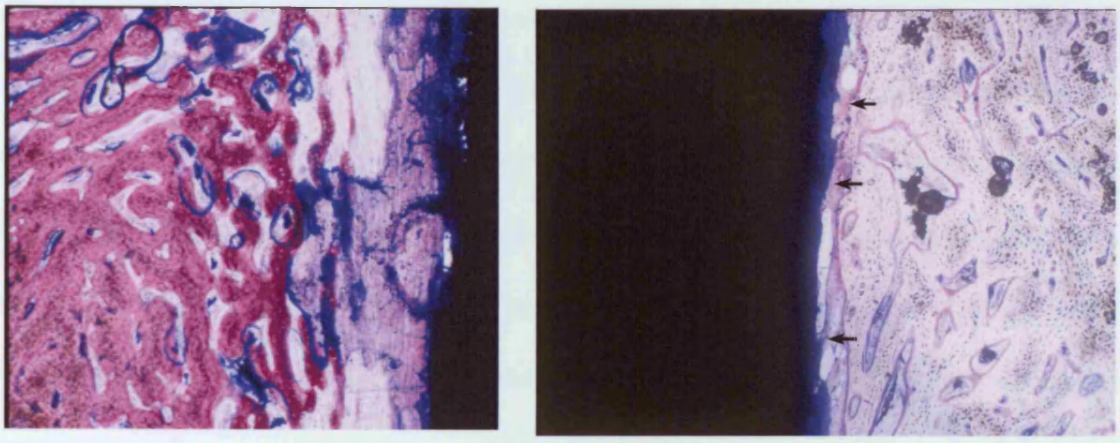
In the Colloss specimen, woven bone appears to surround an area of fibrous type tissue (FT) lying adjacent to the titanium prosthetic surface. It is possible that this represents a remnant of the implanted Colloss.

6.4.3.2. Undecalcified histology 8 week specimens

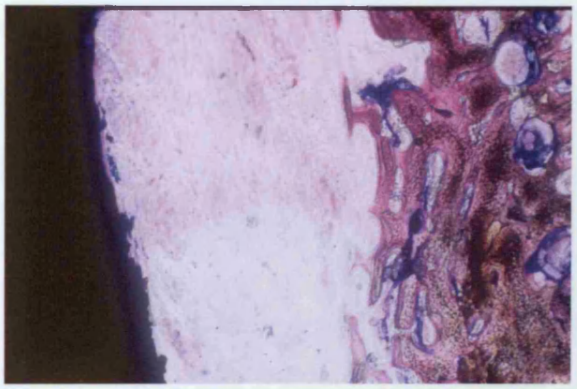


a b

Fig.20a(control) and b(Colloss). 8 week specimens with 1mm periprosthetic defects. The bone filling the defects has matured in both specimens. The woven bone noted in 4 weeks specimen has been partially replaced by lamellar bone in which Haversian systems have formed. Bone in close relationship to titanium implant, but in both control and Colloss remain separated by a thin layer of fibrous tissue (Arrows)



a b



C

Fig.21. a(control), b+c(Colloss). 8 week specimens with 2mm defect. Immature woven bone is present in both control (a) and Colloss specimens (c). There remains a gap between implant and bone.

In specimen (b) which contained Colloss, bone has matured and partially remodelled. This bone lies in close relationship with the implant prosthesis (arrows).

In specimen (c) which contains Colloss there appears to be fibrous type tissue between the new bone trabeculae and the implant surface. This may represent remnants of the Colloss implanted at the time of surgery. Alternatively it may represent fibrous tissue forming secondary to the hosts response to the procedure.

6.5. Discussion

The fixation of uncemented intramedullary stems used in joint arthroplasty relies on their secure fixation to bone. Osseointegration with the ingrowth of bone onto implant surfaces is important to achieve such fixation. The osseointegration of titanium press fit and screw implants in humans and animals has been examined by investigators in previous studies.[188,189,190,191] In these studies direct bone to implant contact was evident in retrieval specimens. Although uncemented hip replacements are implanted with an interference fit, it is likely that periprosthetic gaps remain, particularly following revision surgery where bone loss is inevitable. Methods of enhancing peri-implant bone formation to obliterate such gaps and promote osseointegration will improve implant stability.

Therefore, in this study we have investigated the formation of bone adjacent to non-load bearing titanium implants using a gap model and examined the effects of Colloss on the process of bone ingrowth. The results obtained from the experiments described did not provide evidence to support the study hypothesis. Compared to the control groups Colloss did not enhance the formation of bone within periprosthetic defects, nor did it enhance the osseointegration of titanium implants.

The bone mineral density measurements of the tissue within the periprosthetic defects were consistently greater in the Colloss specimens for 1 and 2 mm gap defects at four and eight weeks. However, a significant difference was not observed. It is possible that differences in bone mineral density would have become evident if the implant was left in situ for a longer period of time.

The presence of Colloss in the periprosthetic defects did not appear to have any deleterious effects on the formation of bone within the defects. New woven bone was evident in both groups at 4 weeks, and by 8 weeks there was no significant difference in the peri-implant gap area between the 1mm and 2mm defects. Eight week defects in

the 1mm Colloss specimens were on average less than those of the control group, but this differerence was not significant. In all cases where new woven bone formation was observed it formed in continuation with the host bone in the defect, towards the implant surface. Bone formation was not initiated at the titanium implant surface, nor did it appear to form de novo within the periprosthetic defect in any of the specimens observed.

The presence of Colloss in 1mm defects did, however, result in a significant reduction in the size of defects' area from the four to eight week time periods. This difference between four and eight week 1mm defect specimens was not observed in the control group. In the majority of specimens, both control and Colloss, a gap was discernable between the implant and host bone. This often contained fibrous tissue.

In some of the Colloss containing specimens there was fibrous type tissue, adjacent to which were the prominent trabeculae of newly formed bone. We were unable to determine the precise nature of this tissue, but it is plausible that it comprises residual Colloss. If this tissue represents Colloss, this may prove to be detrimental by acting as a physical barrier to bone ingrowth. However, this did not become apparent during the eight week duration of the study. In contrast to previous reports of the osseointegration of press fit implants, contact between bone and implant was unusual in this gap model for the study duration. There appeared to be direct contact of new bone with the implant in only one specimen. In this case a 2mm gap had been pre-filled with Colloss and at eight weeks there was a layer of poorly mineralised bone covering the shot blasted titanium implant surface.

Summary

The presence of Colloss within the periprosthetic gap did not enhance bone formation or increase bone mineral density within 1 or 2 mm defects adjacent to shotblasted titanium implants at 4 and 8 weeks following implantation.

7.Chapter 5

General Discussion

The determination of the precise mechanisms of action of Colloss on the growth and differentiation of bone marrow stromal stem cells was beyond the scope of this study. The aims of this study were to establish the effects of Colloss on the behaviour of BMSSCs *in vitro* and its effect on bone formation *in vivo*.

Colloss contains an abundance of collagen type 1 as well as bone matrix proteins, both of which are conducive to the osteogenic process. Bone matrix contains a repertoire of proteins which are involved in the cascade of bone formation. Type one collagen, of which there is an abundance in Colloss, may also have an important role in the osteogenic process. Type one collagen molecules are released from cells as a pro collagen which is then activated extracellularly by cleavage of the carboxy terminal pro peptide. Two thirds of the amino acids are glycine, proline and hydroxyproline. Collagen molecules then combine to form a triple helix, consisting of 2 alpha and one beta chain, which form a right handed triple helix. Glycine is present at every third position, which is important to maintain the helix structure.

Collagen type I is the most abundant protein within the organic matrix of bone, of which it represents 90%. The remaining 10% is comprised of osteocalcin, osteonectin, glycosaminoglycans and lipids. It has a role not only in maintaining the structural integrity of bone, but also has effects on cell behaviour during bone formation. It is not only osteoconductive, but can also bind growth factors which are involved in the process of osteogenesis[192]. It also has binding sites for osteonectin and osteocalcin, which in turn can bind calcium allowing initiation of the mineralisation process.[193, 194] Collagen also has the RGD sequence of amino acids (Arg-Gly-Asp) which interact with the CD44 cell surface receptors allowing interaction between collagen and cells of the extra cellular matrix.[195] Collagen has osteoconductive properties. Collagen based

implants have been used alone or in combination with autologous bone graft, where they have been shown to stimulate bone formation in animal spinal fusion and critical gap defect models.[196,197] In addition to the RGD sequence, collagen has a DGEA cell binding domain comprising the amino acid sequence Asp-Gly-Glu-Ala. In animal studies there is evidence that interactions between bone marrow stromal stem cell surface integrins and the DGEA collagen domain can stimulate the osteoblastic differentiation of these cells.

Muzino et al,[147] examined the behaviour of collagen type I on rat bone marrow cells and the effects of blocking interactions between the alpha 2 beta 1 integrin and collagen. The presence of collagen type 1 stimulated the osteoblastic differentiation of these cells, signified by the expression of Alkaline Phosphatase, osteocalcin, osteopontin and the synthesis of collagen type 1. In contrast, cells cultured without collagen type 1 did not undergo osteoblastic differentiation. When the cell integrin receptors were blocked by the addition of DGEA peptide (the cell binding domain of collagen), the osteoblastic differentiation of bone marrow cells was inhibited. Furthermore, the addition of antibodies to block the alpha 2 integrin also inhibited the osteoblastic differentiation of cells. This suggests that collagen – integrin interactions play an important role in the mediation of extracellular signals required for the differentiation of bone marrow stromal stem cells.

The role of collagen type I on osteogenesis was examined in a further study by Takeuchi et al[198] who investigated the effects of collagen type one on TGF β receptors and ALP expression on rat bone marrow stem cells. The expression of Alkaline Phosphatase in osteoblasts was inhibited by TGF β in long term cultures. It was shown that collagen increased ALP activity in cultures by down regulating TGF β receptors, and that this effect was mediated via the alpha 1, beta 2 integrin. It was also found that collagen fibrils synthesised by the osteoblasts contributing to the formation of

extracellular matrix had the same effect as exogenous collagen on TGF beta receptors, resulting in enhancement of ALP expression. Despite these results it remains unclear whether such mechanisms are applicable to human bone marrow stromal stem cells.

Although Colloss consists mainly of collagen type I, it is also derived from bone matrix which contains several growth factors in low concentration. These include TGF-β, IGF, IL-1, IL6, PDGF, CSF, TNFα, PG's and bone morphogenic proteins. Whilst it is feasible that these factors may have influenced bone marrow stromal stem cell behaviour *in vitro*, it is unclear as to whether the manufacturing processes performed during the production of Colloss impair, or perhaps even inactivate, these growth factors.

Colloss influenced the expression of osteogenic differentiation markers when cultured on hydroxyapatite, titanium and monolayer cultures. Some, but not all, of the osteogenic markers were elevated. With stem cells cultured in monolayer, Colloss stimulated the mineralisation of cultures, evidenced by increased calcium content. In this study we chose concentrations of Colloss of 1:100 and 1:50 dilution. Is is possible that for stimulation of osteoblastic differentiation these concentrations are suboptimal.

When bone marrow stromal stem cells were cultured on titanium, observation of the early peak in Alkaline Phosphatase and increased osteopontin expression on days 14 and 21 supports the hypothesis that Colloss has osteogenic properties. However, in this experiment, the synthesis of collagen type I was not enhanced by the pre-treatment of titanium with Colloss.

When cultured on hydroxyapatite, Colloss lead to an increase in collagen type I synthesis, but not Alkaline Phosphatase or osteopontin when compared to controls. Collagen type 1 expression is not specific to osteoblasts as fibroblasts can also express large amounts of collagen type 1 in vitro. However cell cultures in this study can be distinguished from fibroblastic cultures by their osteogenic marker profiles. Whilst

collagen type 1 can be expressed both by fibroblasts and osteoblasts, proteins with a role in the mineralization phase of osteogenesis such as osteopontin are not expressed or are expressed in negligible amounts by fibroblasts.[199]

A weakness of this study was the small sample number (n) in the cell culture studies and the use of non parametric statistics which was necessitated by the non normal distribution of the data. Both these factors reduce the power of the study to determine differences in osteogenic marker expression between culture groups and within groups at different time periods.

Despite these observations, when implanted in vivo within periprosthetic gaps adjacent to titanium implants, Colloss did not appear to enhance the quantity of bone formation during the 8 weeks prior to implant harvest. The Colloss was, however, biocompatible and did not appear to cause any adverse effects.

Bone marrow contains a mixed population of cells, including committed osteoprogenitors. To ensure the validity of results it is important to ensure that contamination of stromal stem cell isolates with progenitors is minimised. In this study we used methods of stem cell harvest and isolation which are well documented in the literature[122,78]. Ficoll was used to separate bone marrow stromal stem cells from bone marrow using a density gradient technique. Using antibodies to STRO-1 we were able to confirm that we had obtained a population of bone marrow stromal stem cells. Although cell sorting devices were not used, by labelling cells with stro –1 with indirect immunofluorescence, we were able to confirm that cell isolates comprised stromal stem cells. Is is possible that progenitors may have been present within isolates, but the number is likely to be proportionally insignificant. Furthermore, stem cells used for control and experimental groups were derived from the same primary isolate, hence minimising the risk of bias.

7.1. Conclusions

Colloss does not enhance bone formation or increase mineral content of new bone within defects adjacent to non-loaded titanium implants in a sheep gap model. Colloss suspension appears to have an effect on the expression of osteogenic markers by bone marrow stromal stem cells cultured in monolayer, smooth polished titanium and hydroxyapatite granules. In each case, some but not all of the osteogenic markers were expressed at levels above those of the control group. Therefore, with regards to cell culture *in vitro*, the results, although supportive of the osteogenic potential of Colloss, are inconclusive.

7.2. Further work

It is likely that the influence of Colloss on growth and differentiation of bone marrow stromal stem cells is concentration dependent. The optimal concentration of Colloss to stimulate cell differentiation is unknown. Further study of the effects of Colloss at various concentrations on stem cell behaviour may allow this to be determined.

Further information on the effects of Colloss on the expression by BMSSCs of mRNA's which encode for osteogenic markers such as osteocalcin, osteopontin, bone sialoprotein and osteonectin, could be obtained by utilising RT PCR techniques. This would allow quantification of gene expression and determination of the sequence of gene expression, allowing direct comparison with control cultures.

Colloss did not appear to enhance bone formation within periprosthetic defects in this study. It would be of interest to determine whether it can stimulate bone formation in the absence of bone defects. This could be assessed by applying Colloss to press fit titanium implants

7.3. Acknowledgements

Professor Gordon Blunn, head of department, Centre for Biomedical Engineering, Stanmore, Midddlesex

Ossacur AG, Germany for suppling Colloss and funding consumables

Gilllian Highes, Royal Vetinary College, South Mimms, Hertford

Mr T.W.Briggs, Consultant Orthopaedic Surgeon, the Royal National Orthopaedic Hospital, Stanmore, Middlesex

Catherine Pendegrass and Oscar Lee for tuition in cell culture techniques

Michael Kayser for assistance with Scanning electron microscopy

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