CHARACTERISATION AND BIOCOMPATIBILITY EVALUATION OF CALCIUM PHOSPHATE BIOMATERIALS IN VITRO

BY

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A thesis submitted in accordance with the regulations governing the award of the Degree of Doctor of Philosophy in Bioengineering

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ABSTRACT: Medical applications of calcium phosphate biomaterials are limited because of poor mechanical properties and acute inflammation reactions which take place occasionally in the clinic. To increase the usefulness of calcium phosphate biomaterials it is necessary to improve the mechanical properties and biological character. Processing and characterization of porous hydroxyapatite (HA) and dense composite (HA-Spinel) biomaterials have been performed in the present research. Biocompatibility of these biomaterials has been examined in vitro using human and rat immortalized osteoblast cells, and the advantages and limitations of cell culture biocompatibility tests are discussed. X-ray analysis of material structure demonstrated that after sintering at 1450°C, HA-Spinel was changed into tricalcium phosphate (TCP)-Spinel phase structure. Mechanical properties testing showed that the bending strength and compressive strength of HA may be improved by adding Spinel. Biocompatibility examination demonstrated that both human and rat osteoblast cells anchored to the surface of the porous and dense biomaterials in a short time, and subsequently, grew and proliferated normally on the surface of these biomaterials. Cytotoxicity evaluation in vitro by studying material extracts demonstrated that compared with the control group of cells cultured on polystyrene, HA-Spinel possessed slight toxicity. Cell growth in HA-Spinel first extracts was slightly impaired. Tritium labeling and immunofluorescent analysis proved that human osteoblast cells and rat osteoblast cells have normal expression of collagen synthesis on the above biomaterials. Confocal laser scanning microscopy (CLSM) observation showed that collagen fibers were produced on these materials, and the amount of the collagen synthesized on the materials increased with culture time. Subsequent analysis indicated that both HA and HA-Spinel can strongly adsorb serum and albumin proteins from culture media and the amount of protein adsorption was proportional to the porosity in the materials. Protein adsorption on the material surface was saturated usually in 2-4 hours, and 1/3~1/2 of the total protein adsorption was achieved in several minutes. In vitro assay also confirmed that human and rat osteoblast cells can be applied as an in vitro model to evaluate the biocompatibility, cytotoxicity and other biological characteristics. Compared with human osteoblast cells, rat osteoblast cells have a greater proliferation rate. In normal conditions, the proliferation rate of the rat osteoblast cells is 2-4 times that of the human osteoblast cells and for this reason rat osteoblasts seem more sensitive to material extracts.

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DEDICATION

To the greatest mum and dad in the world
and
To the great friendship between British people and Chinese people

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ABBREVIATIONS

ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
BG	Bioglass
BHI	Brain heart infusion
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscopy
CPO	Critical point drying
DMEM	Dulbecco's modification of Eagles medium
DMSO	Dimethylsulphoxide
FCS	Foetal calf serum
HA	Hydroxyapatite: $Ca_{10}(PO_4)_6(OH)_2$
HA-Spinel	Hydroxyapatite-Spinel: $Ca_{10}(PO_4)_6(OH)_2$-$MgAl_2O_4$
IGF	Insulin growth factor
ISO	International Standards Organization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCP	non collagenous protein
OPT	O-phthaldehyde
PBS	Phosphate buffered saline
PEST	Penicillin Streptomycin
PTH	Parathyroid hormone
PTM	Phase contrast microscope
RGD	Arginine-glycine-aspartic acid
SAB	Sabouraud's liquid
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TCP	Tricalcium phosphate: $Ca_3(PO_4)_2$
TCPS	Tissue culture polystyrene
TEM	Transmission electron microscopy

Chapter 1 Review of the literature

1.1 Introduction

Skeletal reconstruction is required in cases involving large defects created by tumour resection, trauma, and skeletal abnormalities. A number of techniques for promoting bone repair have been reported, and most common clinical approaches utilise allografts or autografts. Grafts and flaps of autogenous tissue are two of the most successful means of reconstruction because they allow for the transplantation of bone containing bioactive molecules, and frequently, a vascular supply that will allow the transplant to survive and remodel even in hostile radiated environments (Damien and Parson, 1991). Unfortunately, although the use of autograft material is the preferred technique at this time, only a minimal amount of tissue can be harvested for the autograft, and it can be very difficult to form into the desired shape(Ravaglioli and Krajewski 1992). Therefore, there are restrictions in its use. These restrictions include donor site mobility, limited donor bone supply, anatomic and structural problems and elevated levels of resorption during healing. Allografts have the potential of transferring pathogens and other current therapies involve the use of allograft bone, nondegradable bone cement, metals, polymers, and ceramics (Makoto et al, 1988; Park et al, 1992).

In the treatment of disease or injury it has been found that a variety of nonliving materials are of use, which are called biomaterials. Common place examples include tooth fillings and artificial joints. A biomaterial is a synthetic material used to replace part of a living system and to function in intimate contact with living tissue. The uses of biomaterials include replacement of a part of the body that has lost function due to disease or trauma, to assist in healing, to improve function, and to correct abnormalities. The role of biomaterial is influenced considerably by advances in many areas of medicine and also influenced markedly by the tissues that surround the materials. For example, with the advent of antibiotics, infectious disease is less of a threat than in former times. On the other hand, all of these options have their associated problems and limitation (Benahmed, 1996; Park et al, 1992). These short comings have inspired a search for improved methods of repairing skeleton defects. The performance of biomaterials in the body can be viewed from several perspectives. We can assess the interaction of

biomaterials in vitro at the cellular level, or at an organ level. The role of a biomaterial is governed by the interaction between the material and the body, especially, the effect of the tissue environment on the material. Following further advances in materials science and technology, more and more artificial materials will be applied in the clinic to deal with bone disease. The success of a biomaterial in the human body depends on a lot of factors, such as the material design and properties, the location in the body, biocompatibility, as well as the condition of the patient. There are multiple modes of material failure, which include failure introduced by the intrinsic character of the materials, such as composition and structure, and failure caused by the effect of the material upon the body, such as toxicity, irritation, and inflammation.

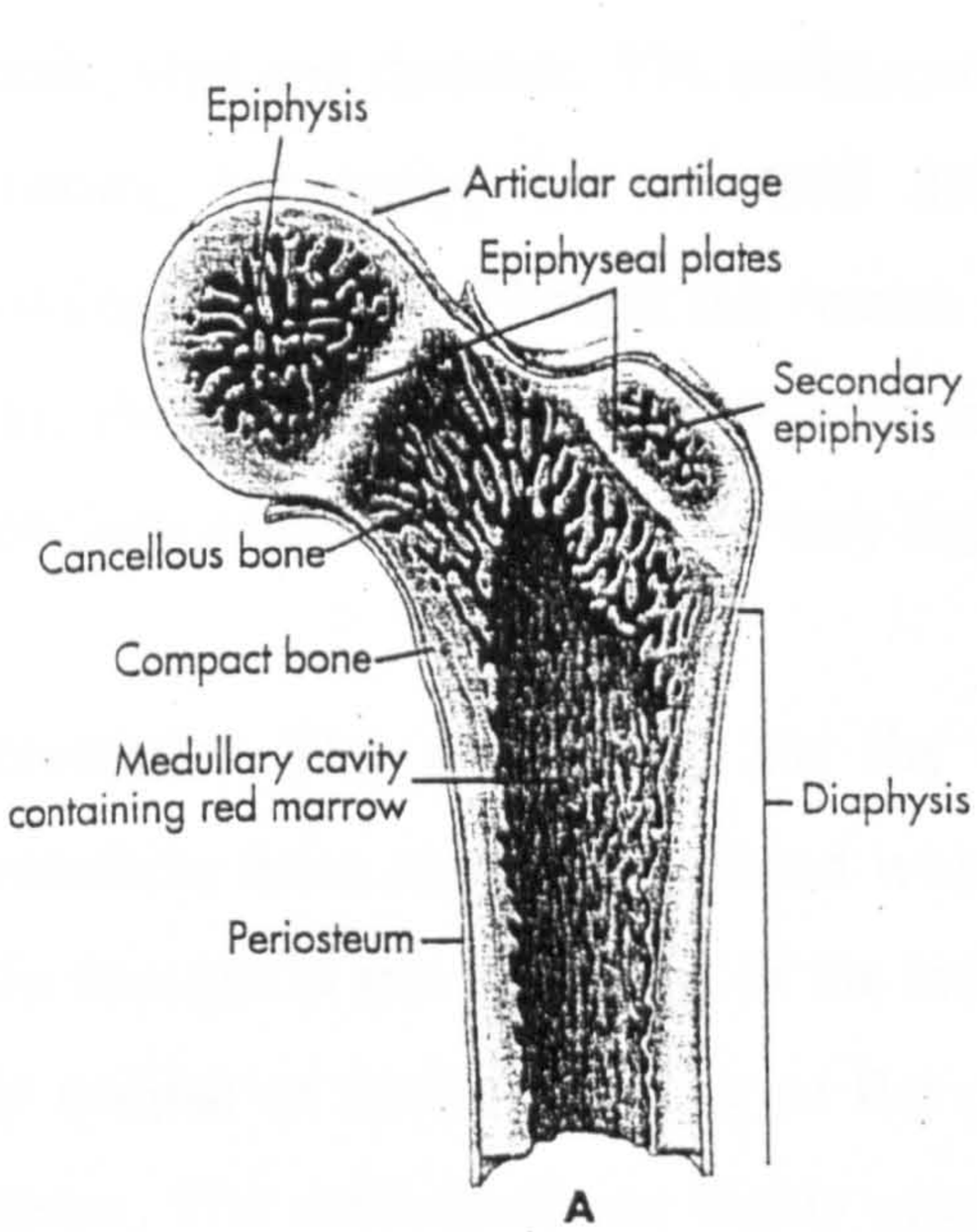
Biocompatible materials should not irritate the surrounding structure, nor provoke an inflammatory response. The ideal bone substitute would approximate the autograft, requiring minimally that it be biocompatible and conductive, contain osteoconductive factors to enhance new bone ingrowth and contain osteogenic cells to begin secreting new extracellular matrix. Bone regeneration by transplanted autogenous osteoblasts meets these requirements and thus holds promise as an improved method of skeletal reconstruction. The bone substitution materials used in this approach must allow for attachment of osteoblasts, because they are anchorage-dependent cells that require a supportive matrix in order to survive. At the least, the bone substitute biomaterial must provide an appropriate environment for the proliferation and function of osteoblasts, and allow for the ingrowth of vascular tissue to ensure survival of the transplanted cells. Finally, if necessary, it should be biodegradable with a controllable rate of degradation into molecules that can easily be metabolised or excreted. The bone substitution materials must satisfy the requirements of the surrounding tissues or organs, and so it is important to understand the basic structure and function of the bone.

1.2 Construction of bone

Bone structure: Mature bone consists of a central fatty or haematopoietic marrow that is supported and surrounded by bone tissue and periostium. Marrow can serve as a source of bone cells and disorders or mechanical disruption of the marrow can affect the activities of the bone and periosteal cells (Buckwalter et al, 1995). Close

inspection shows that there are two forms of bone tissue: cortical (compact) bone and cancellous (trabecular) bone. Cortical bone and cancellous bone have the same matrix composition and structure, but the mass of the cortical bone matrix per unit of volume is much greater. Cortical bone has less porosity (approximately 10 per cent porosity) than cancellous bone (50-90 per cent porosity). Figure **1.1** shows the structure of human long bone which consists of cortical bone and cancellous bone. As the compressive strength of bone is proportional to the square of its density, the modulus of elasticity and the ultimate compressive strength of cortical bone may be as much as ten times greater than that of a similar volume of cancellous bone (Buckwalter and Cooper, 1987; Men et al, 1989). Cancellous bone has approximately twenty times more surface area per unit of volume than does cortical bone, and its cells lie primarily between lamellae or on the surface of the trabeculae. Cortical bone forms approximately 80 per cent of the mature skeleton and surrounds the marrow and the cancellous bone. Bone may be regarded as a composite of collagen fibres and an inorganic matrix, mainly hydroxyapatite (HA) (Mundy et al, 1993). During bone formation, the first step is the generation of collagen fibres, followed by mineralisation as a second step a few days later (Roberts et al, 1992). Bone consists of 22% w/w organic matrix, 72% w/w inorganic matrix, and 2% w/w water. With the density of HA at 3.14~3.16 g/cm^3, and that of the organic matrix about 1g/cm^3, the volume fractions of the organic and inorganic matrix are about the same. The inorganic matrix is imbedded in a fibrous frame of collagen. The collagen fibres provide the high tensile and bending strength of bone, whereas the inorganic matrix is responsible for the high compressive strength and the stiffness of bone (Silver, 1994; Cowin et al, 1989; Yano et al, 1990). The human skeleton is subject to a continuous series of metaplastic processes during which bones are formed in accordance with the given physiological requirement (Martin et al, 1990; Ravaglioli and Krajewski, 1992). Human body employs two special kinds of cells for carrying out metaplastic process: those which serve as bone forming cells-**Osteoblasts** and those which dissolve unwanted bone—**Osteoclasts**. During the life of the human individual, these two kinds of cells always act according to the physiological requirements. It has been quite clearly demonstrated, especially in the case of calcium phosphate ceramic biomaterials embedded in connective tissue that the osteoclasts are activated, i.e., they dissolve the

ceramic enzymatically as if it were bone (Yasemski et al, 1996; Nordsletten et al, 1996; de Bruijn et al, 1994).



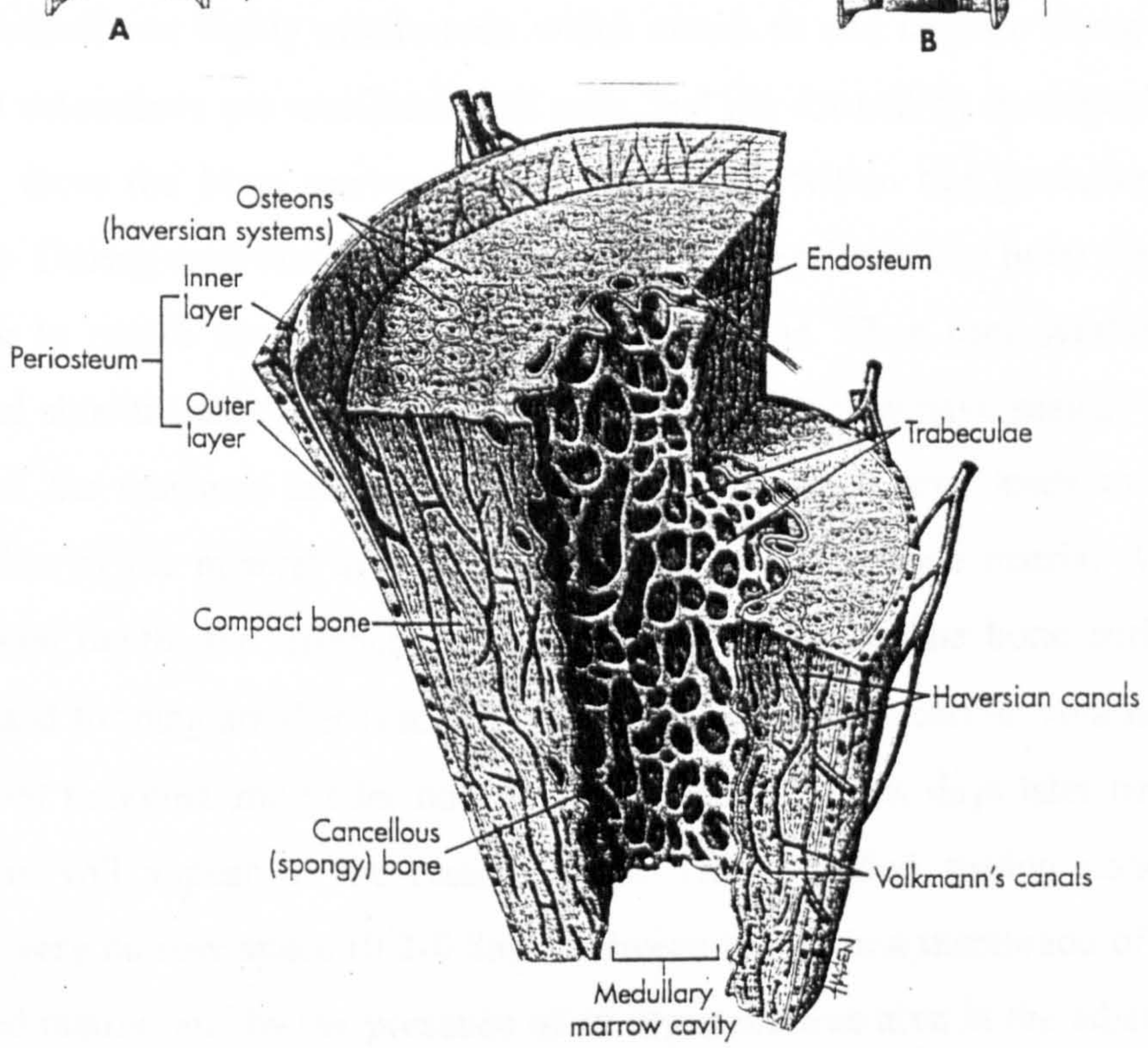


Figure 1.1. Structure of a long bone. A femur showing epiphysis, epiphyseal plates and diaphysis (A); A femur showing cancellous bone and compact bone (B); and the inner features of a portion of a femur (C). (From Seeley et al, 1995).

1.3 Physiology of bone

Bones and teeth are the only structures within the body where calcium phosphate provides the basic functional structures. Despite their calcified nature, both the tissues are organic, vital and dynamic. The maintenance and renewal of bone is a physiologic marvel of nature, but sadly, the structural defects to teeth can only be renewed by non physiological means. Though the reason for such active bone turnover is still not clear today, the approaches and observations in vivo and in vitro have demonstrated that both osteoclasts and osteoblasts play a very important role in bone turnover and repair.

Osteoclasts: The osteoclasts are the cells responsible for the resorption of the extracellular bone matrix. Integrated with osteoblasts, osteoclasts play an important role in the changes in size and shape of the individual bones during growth, and in bone repair after trauma or fracture leading to the quantitative and qualitative maintenance of the skeleton. The osteoclasts are highly motile cells which attach to and migrate along the bone surface. Most osteoclasts are multinucleated cells, and are formed by mononuclear precursors derived from the bone marrow and differentiated within the granulocyte-macrophage lineage. During resorbtion, osteoclasts attach to the mineralised bone matrix that they are going to resorb by forming a tightly sealing zone. Then they synthesise several enzymes and simultaneously lower the pH of the area that they have sealed. The concerted action of the enzymes and the low pH in the resorbed area leads to the extracellular digestion of the mineral and the organic phases of the bone matrix. After resorbing to a certain depth, the osteoclasts detach and move along the bone surface before reattaching and forming another resorption pit. In the process, a certain volume of bone matrix has been removed and under normal circumstances, a few days later newly formed bone matrix will appear at the resorbing pit. The so called sealing zone is characterised by a very narrow space (0.2-0.5nm) between the plasma membrane of the cell and the calcified matrix and by the presence of an organelle-free area in the adjacent cytoplasm. Depending on the behaviour (walking, sitting and resorbing) of the osteoclast movement there are different podosomes in the sealing zone. In terms of composition and function, podosomes and focal adhesion plaques are clearly related but there are important functional differences. In contrast to focal adhesion plaques, podosomes are

less tightly associated with the substratum and are highly dynamic, changing their size and have life spans of 2-12min. In highly motile osteoclasts, few podosomes are observed and seem to be confined to the irregularly shaped leading edge of the cell. Upon arrest of movement and attachment, numerous podosomes are formed and organised into a peripheral ring. Rapidly thereafter, the seal is established and the podosome structure is replaced by two concentric rings in which the podosome density cannot be individually observed (Lakkakorpi and Vaananen 1991).

During the attachment to substratum, the transmembrane proteins which are members of the integrin family of adhesion molecules with specific, receptor-like, extracellular binding sites, mediate cell-substratum and cell-cell interaction. By recognising the arginine-glycine-aspartic acid (Arg-Gly-Asp: RGD) sequence these transmembrane proteins bind the osteoclasts to the substratum, or conjugate with each other. The attachment area of the osteoclast membrane is recognised as a ruffled border and constitutes the resorptive territory where enzymatic breakdown of bone surface occurs. A sealing zone(the zone of attachment) between the ruffled border and bone surface isolates the micro environment, and osteoclastic anhydrase lowers the pH, allowing solubilization of calcium-phosphate, and exposing the organic components of the matrix. The resorption sites(pits) are referred to as scalloped resorption lacunae. Pits become the sites for bone formation within a couple of days. The coupling of resorption with bone formation is part of the remodelling aspect of bone. It is hypothesised that coupling is mediated by factors secreted by osteoblasts which then stimulate osteoclasts. Osteoclast activation has been studied extensively using cells isolated from new-born rat bone and plated onto thin slices of cortical bone. Resorption is quantified by measuring the areas or numbers of resorption pits produced by osteoclasts in response to treatment with bone-resorbing agents (Martin et al, 1993).

Calcitonin which causes loss of the peripheral band of the podosomes directly inhibits bone resorption after binding to receptors present on the osteoclast basolateral membrane. In contrast, retinal and retinoic acids stimulate bone resorption by promoting podosome formation. Malgaroll et al. (1989) have shown that inhibition of bone resorption is associated with an elevation of cytoplasmic Ca^{2+} levels after calcium treatment. By reducing intracellular calcium, in contrast, the formation of podosomes is

promoted. Furthermore, the binding of RGD-containing matrix proteins to their integrin receptors induces a decrease in intracellular Ca^{2+} and activates bone resorption.

Osteoblasts: Osteoblasts are histologically recognised as plump, cuboidal, mononuclear cells lying on the matrix which they have synthesised. The gap junctions which connect them to their neighbours and to adjacent bone-lining cells provide a mechanism of intercellular communication likely to be important in their function. They have a prominent Golgi apparatus and a basophilic cytoplasm rich in endoplasmic reticulum reflecting their capacity for protein synthesis. They are rich in alkaline phosphatase (ALP) (ALP activity was used as a marker for stromal cell differentiation) and synthesise predominantly type I collagen and certain other proteins including osteonectin, osteocalcin, etc. They respond to several hormones, and a number of growth factors (Petite, 1996; Ong et al, 1998; Kieswetter et al, 1996). Osteoblasts originate from the stromal fibroblastic or osteogenic system of the bone marrow(Friedenstein, 1976). By bone marrow stromal cell differentiation, fibroblasts and osteogenic precursor are derived which further differentiate into osteoblast precursors, osteoblasts, lining cells and osteocytes. The most apparent function of osteoblasts is the synthesis and secretion of the organic matrix of bone (Nufussi et al, 1985). The generic term osteoblast is commonly used to cover all mature members of the osteoblast lineage, i.e., osteoblasts, lining cells, and osteocytes. The osteogenic potential of the population is demonstrated by the formation of nodules which contain multiple layers of cells laying down an organised extracellular matrix consisting predominantly of collagen that subsequently undergoes mineralisation(Bellows. 1986). According to Stein et al. (1989), the entire developmental sequence can be divided into three distinct stages. The initial proliferation stage(days 0-15) is characterised by the synthesis of an organised bone-specific extracellular matrix. The shutdown of proliferation is followed by the stage of matrix maturation-procollagen or collagen synthesis(days16-20), which renders the matrix competent for the final stage of mineralization. Osteoblasts synthesise collagen-rich matrix which mineralises to form mature bone. Approximately 90% w/w of the organic matrix of bone is collagen, and almost all of this is type I collagen (Pasquier et al, 1996). Accordingly, type I collagen represents more than 85% w/w of the total collagen expressed by normal or transformed osteoblastic cells in culture. Many factors influence the mineralisation process, and in this

context, a number of non-collagen proteins of bone have been recently characterised. For example, osteonectin is a protein capable of high-affinity binding to both HA and collagen, and it may facilitate mineralisation of type I collagen(Termine, 1981,). More recently, Holland et al (1987) demonstrated that osteonectin in nonmineralised tissue may play a role in suppressing the rate of HA formation. 1,25-Dihydroxyvitamin D_3 is the most active metabolite of vitamin D and is essential for bone mineralisation. Receptors for 1, 25-Dihydroxyvitamin D_3 are present in osteoblasts and it can alter alkaline phosphatase activity as well as the expression of matrix proteins such as collagen(Harrison et al, 1989), osteopontin, osteocalcin, and matrix gla-protein in these cells. Owen et al. (1990) showed that 1,25-dihydroxyvitamin D_3 can both positively and negatively regulate expression of osteoblast phenotypic markers such as type I collagen, osteopontin etc. When 1,25-dihydroxyvitamin D_3 was added to cultured osteoblasts from day 6 to day 34, subsequent mineralization was inhibited and osteocalcin mRNA was not expressed. On the other hand, treatment initiated at day 20 after the phase of matrix maturation resulted in enhanced osteocalcin mRNA expression. However, systemic hormones, including parathyroid hormone and local cytokines, may stimulate osteoblasts to release mediators that activate osteoclasts.

1.4 Extracellular Matrix

Organic bone matrix: Bone matrix is a composite material consisting of an organic and an inorganic component (Glimch, 1992). The organic component usually contributes a little more than 20 per cent of the wet weight (Triffit, 1980; Buckwalter et al, 1995). The organic matrix of bone resembles the matrix of dense fibrous tissues such as tendons, ligaments, and joint capsules (Yao et al, 1991). Collagens, predominantly type I, along with small amounts of types V and XII, make up approximately 90 per cent of the organic matrix. The other 10 per cent consists of non-collagenous glycoproteins and bone specific proteoglycans (Raisz and Kream, 1982). Type I collagen is distinguished from the other collagens by its unique amino-acid content, the relative large diameter of its fibrils, and its presence in tissue subjected to large tensile loads, including tendon and ligament (van der Rest Michel 1991). Bone also contains a variety of non-collagenous proteins that may influence the organisation of the matrix, the mineralisation of the bone,

and the behaviour of the bone cells. These proteins include osteocalcin, osteonectin, bone sialoprotein, etc. (Boskey 1989, 1992). Bone matrix also contains growth factors that can influence the function of bone cells. Growth factors that have been identified in bone include the transforming growth factor-β family, insulin-like growth factor-1 (IGF-1), insulin-like growth factor-2 (IGF-2), bone morphogenic proteins, platelet-derived growth factors, interleukin-1, interleukin-6, and colony-stimulating factors(Ingram et al, 1994; Chesmel et al, 1992). Their presence within bone and their potential to affect the activity of bone cells strongly suggests that they have an important role in controlling bone-cell function (Nordsletten et al, 1996).

Inorganic bone matrix: The inorganic component contributes approximately 65 per cent of the wet weight of the bone (Buckwalter et al, 1995). The inorganic matrix, or mineral phase of bone performs two essential functions: it serves as an ion reservoir, and it gives bone most of its stiffness and strength. About 99 per cent of the body calcium, about 85 per cent of the phosphorus, and between 40 and 60 per cent of the total body sodium and magnesium are associated with the bone mineral crystals, the major source of these ions for supply to the extracellular fluid. By serving as a reservoir for these ions, the inorganic matrix of bone helps to maintain their extrcellular fluid concentrations within the ranges necessary for critical physiological functions, including nerve conduction and muscle contraction as well as most of the important biochemical reactions. Calcium phosphate crystalises with the mechanical properties necessary to withstand the forces imposed by normal activity. Early research claimed that bone was HA ($Ca_{10}(PO_4)_6(OH)_2$)(Hench, et al, 1971; Hulbert, 1978;). Holliger and McAllister, (1995) have shown that during osteogenesis(bone formation), amorphous forms of calcium phosphate deposited first, maturing to crystalline HA, but some studies of the bone mineral crystals have shown that they are not pure HA (Ducheyne, 1993). Instead, they contain both carbonate ions and acid phosphate groups (HPO_4^{-2}). Ducheyne et al (1993) and de Groot (1990) suggested that unlike pure HA, bone crystals do not contain OH groups, therefore, bone mineral crystals should be classified as apatite rather than HA. However, in my early studies, we still found that there is common crystalline structure between human femoral bone and not well-crystallised HA (Ruan et al, 1993). Bone mineral crystals undergo important changes in composition, especially in the concentration of carbonate and acid phosphate

groups as a function of the age of the crystals. Thus, the biological functions of the crystals, including their role as ion reservoirs and their effects on cell function, and possibly their contributions to the mechanical properties of bone, depend not only on the amount of mineral present but also on the age of the mineral crystals. HA is a plate-like crystal measuring 20-80nm by 2-5nm. The inorganic matrix fulfils a significant functional and physiological role. Functionally, it provides rigidity to bone, allowing us to walk upright. It serves as the origins and insertions for muscles, and the ingeniously engineered system has developed sufficient power for locomotion. Physiologically, the inorganic matrix provides life sustaining, serum aliquots of minerals needed for homeostasis, and cellular regulation, and in particular serum calcium is closely titrated.

Mineralisation: The formation of solid calcium phosphate from soluble calcium and phosphate in the organic matrix of bone does not occur as a result of a chemical reaction. It represents a phase transformation, a process exemplified by the formation of ice from water. Solid calcium phosphate in bone first appears as a poorly crystalline apatite. With time, the crystallinity of apatite is increased, but it never approaches the highly crystalline state of naturally occurring geological HA made by the precipitation of calcium phosphate in vitro. Mineralisation in bone matrix occurs in an organised fashion. During the mineralisation of bone, granules of calcium phosphate appear in osteoblast mitochondria and matrix vesicles, extracellular membrane-bound structures formed from the osteoblast plasma membrane (Ohgushi et al, 1996). Although it has been suggested that mitochondria and matrix vesicles may influence mineralisation directly or indirectly by concentrating and releasing calcium phosphate, their exact role has not been established clearly. Mineralisation in the vesicles usually proceeds quite rapidly once it begins; 60 per cent or more of the ultimate mineral forms within hours. After this initial phase, mineral continues to accumulate over a prolonged time, gradually increasing the density of the bone. Although the changes in the composition of the organic matrix that occur during mineralisation remain poorly understood, it is clear that, as mineralisation proceeds, the water and probably the non-collagenous protein concentrations decrease as the mineral concentration increases, but the collagen concentration and organisation remain relatively unchanged. With increasing mineralisation of the matrix, and maturation of the bone crystals, the stiffness of bone increases.

A mechanism of bone tissue mineralisation suggests that mineralisation, i.e. HA formation is performed in collagen via the matrix vesicles (Sauer and Wuthier, 1988; Sautier et al, 1993). Matrix vesicles are tiny(0.1-0.2um in diameter), extracellular, membrane-derived particles that are found selectively at sites of mineralisation in the matrix. The first crystallites of HA mineral are seen within matrix vesicles, often in association with the inner surface of the vesicle membrane. Vesicles near the mineralisation front accumulate increasing numbers of HA crystallites and begin to show penetration of the vesicle membrane by rigid crystal profile. The process of vesiculation and vesicle release is a common phenomenon in nature, and is often related to a time in the cell cycle, postmitosis, but does not require cell death or disintegration. Osteoblasts appear not to undergo programmed cell death after vesiculation (Mardin et al, 1993). One feature common to matrix vesicle formation in all skeletal tissue is the polarised nature of the vesiculation process. Only mineral-facing surfaces of osteoblasts release matrix vesicles (Akisaka et al, 1986). Calcium-depositing activity of matrix vesicles can be quantified by measuring the amount of ^{45}Ca deposited. Anderson et al (1993) demonstrated that mineralisation performed on matrix vesicles consisted of two phases. Phase I is the period during which the first mineral is formed, primarily along the inner leaflet of the matrix vesicle membrane. Ca^{2+} is attracted to the matrix vesicle by the calcium-binding phospholipids and by the various calcium binding proteins(for example; type X collagen). Phase 2 is the period during which the local PO_4^{3-} increases accomplished by the activity of phosphatases, including ALP, which resides in the vesicle membrane. Phosphatase activity increases as the matrix vesicles approach mineralisation. The rate of phase 2 calcification is controlled by matrix components which can accelerate or retard mineral propagation. The rate of HA nucleation is governed by extracellular factors; collagen serves as the substratum into which HA crystals are deposited, providing a favourable environment for HA nucleation and propagation after mineralisation has entered the extra-vesicular phase(Arsenault et al, 1989).

1.5 Bone turnover and repair

Usually the healing time for a simple fracture is in excess of 4 weeks (Damien and Parson, 1991). In the processes of bone turnover and repair, both osteoclasts and

osteoblasts are involved. It seems that osteoclast activation is associated with osteoblast activity, though the activation mechanism is not clear. A possible pathway for the recruitment of osteoclasts is an indirect effect mediated by osteoblasts and perhaps other cells of marrow stroma. However, this remains a controversial question, and some published data suggest a direct effect of bone-resorbing hormones on osteoclast precursors, and in some research marrow cells/monocytes are directly used as osteoclasts to resorb calcium phosphate as well as bioglass materials (Ross et al, 1996). Takahashi et al (1986) noted that osteoclast formation took place in close apposition to clusters of osteoblast-like cells in vitro, raising the possibility that osteoblasts were contributing to osteoclast formation. Cultures of spleen cells can be used as a source of osteoclast precursors. Osteoclast precursors can be formed by co-culture of spleen cells and osteoblasts, and furthermore osteoblast cells are particularly effective at promoting osteoclast formation, which implies that cell-cell contact may be necessary for promotion of osteoclast formation by the osteoblast or stromal cells. Researchers today favour the idea that osteoblasts/stromal cells are required to promote osteoclast formation and the ability of osteoblast-like cells and certain marrow stromal cell lines to promote osteoclast formation suggests that this property is likely to occur in cells throughout the osteoblast lineage (Martin et al, 1993).

Coupling of resorption to formation: A number of observations have recognised that the processes of bone resorption and bone formation are coupled, which means that once bone resorption has occurred, osteoblasts will be in action by making more bone matrix. Figure **1.2** shows the coupling action between osteoclasts and osteoblasts. Throughout the ageing of human bone, it is essential that a balance be achieved between resorption and bone formation. It has generally been accepted that any change in one of those parameters results in a change in the other. A possible mechanism for the coupling of bone resorption and bone formation is that resorbing bone produces a factor which influences the rate of osteoblast activity (Raisz and Shoukri, 1993). The evidence has demonstrated that this coupling process could be mediated by a number of factors such as parathyroid hormone (PTH), and IGF-I. The bone-resorbing hormone, PTH, promotes the synthesis of IGF-I by osteoblasts(Anderson and Morris, 1993). IGF-I is an important

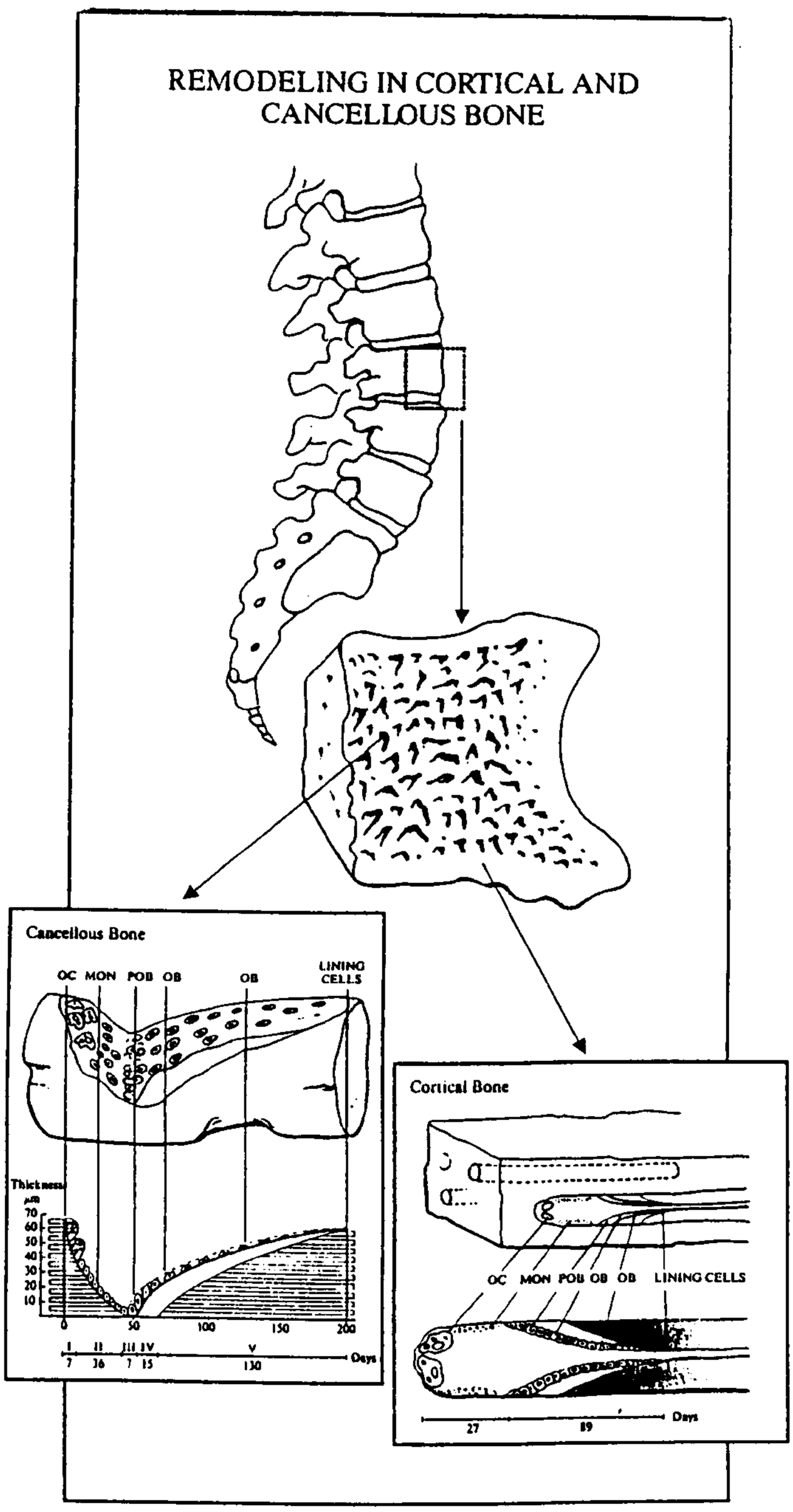


Figure 1.2. Coupling of bone cells. In cortical bone the multicellular unit (BMU) consists of a tunnel drilled out by advancing osteoclasts (OC) followed by mononuclear (MON) cells. Bone formation is initiated by the emergence of preosteoblasts (POB) in the BMU. Later on preosteoblasts differentiate into osteoblasts (OB) that form matrix, which is subsequently mineralised. In cancellous bone the sequence of the events is identical, except for the fact that the process takes place on the trabecular surface. (From Eriksen et al, 1993).

growth factor in bone and promotes the synthesis of type I collagen as well other protein components(Urist et al, 1983). This interaction could therefore provide a mechanism of coupling of bone resorption and bone formation (Canalis et al. 1988, Heldin and Westermark, 1987).

Factors that regulate bone formation and resorption: Bone formation and resorption, the two major processes of bone remodelling, are regulated by defined agents and mechanisms. The formation of new bone consists of the production of a new bone matrix by the osteoblasts. Since the formation of new bone is a function of osteoblast, any factors which can effect the function of osteoblasts will enhance or inhibit bone formation. A number of studies have revealed that bone formation is controlled by systemic hormones and local factors which directly act on osteoblast cells (Cheung an Haak, 1989; Guicheux et al, 1991; de Bruijn et al, 1994). Mediated by local factors,

systemic hormones are likely to have direct effects on bone formation (Cardona et al, 1992; de Bruijn et al, 1994; Nishiyama et al, 1996; Guicheux et al, 1998;). The replacement of small volumes of old bone by new tissue throughout adult life is brought about by the continued repetition of a cyclical process of excavation and repair, each episode extending over $0.2mm^2$ of bone surface and lasting for a period of 4-6 months(Parfitt et al, 1993). At any time about 10%-20% of the bone surface area is involved, and all the surface is involved every 2-5 years, which is the average time interval between successive activation events at same location (Parfitt et al, 1993). In the process of coupling of bone formation and resorption, the first step is the local activation of a segment of the quiescent bone surface. In the second step, the lining cells retreat to expose the bone; and in the third step, osteoclasts attach to the surface by chemotaxis to congregate and fuse (Buckwalter et al, 1995). There follows by erosion development of a microscopic cavity about 50um in depth within a few weeks This then disappears and in the last step, osteoblasts assemble within the cavity and refill in more or less completely over another few months. Unlike resorption, bone formation occurs in two successive stages. Matrix synthesis begins first and determines the volume of new bone but not its density. Mineralisation begins about 10 days later and determines the density of new bone. The rate of bone turnover is determined by the rate of remodelling activation and

the local surface to volume ratio. For a total skeletal calcium of 30mol, this corresponds to about 8 mmol/day (Rodan,1992).

Regulation of bone-cell activity: Bone-cell function is regulated at both the systemic and local level (Russell et al, 1990; Canalis et al,1993b). The genome and systemic hormones control bone-cell function throughout the skeleton. Molecules released from bone matrix, bone cells and their sub-populations, for example cytokines, exert local effects on bone cells. Local factors, including mechanical loading, electrical stimulation, cytokines, and other mediators, may have the most direct effect on bone-cell activity. A critical observation shown that not only is there a clear relationship between bone structure and loading but also living bone adapts to alterations in loads by changing its structure (Buckwalter et al,1995). Furthermore, maintenance of normal bone density requires repetitive loading. Bone electrical phenomenon is contributed to the polar structure of collagen molecules which are oriented in bone. Bone healing and repair could be aided by external electrical stimulation (Park and Lakes,1992). Systemic hormones, such as PTH, calcitonin, and vitamin D can affect the level of serum calcium(Aloia, 1988). Thyroxine, glucocorticoids, and oestrogens influence bone-cell function. PTH, a single-chain polypeptide secreted by chief cells of the parathyroid gland, raises the level of calcium in extracellular fluid, increases the release of calcium from bone, and promotes osteoclastic resorption of bone, by causing bone-lining cells to retract, allowing osteoclasts to gain access to the mineralised surface of the bone and have inhibitory effects on osteoblasts (Mundy, 1993; Kurihara et al, 1991;). Calcitonin, a polypeptide synthesised by C cells of the thyroid gland, has the function to lower the levels of serum calcium by inhibiting osteoclastic resorption of bone. This may occur by inhibition of the formation and activation of osteoclasts (Chambers and Moore, 1983). Also calcitonin may cause osteoclasts to withdraw from the surface of the bone and to divide into mononuclear cells (Fisher and Termine, 1985). The actions of Vitamin D are complex and are the subject of some controversy (Raisz and Kream,1989). The primary role is to increase the level of serum calcium by promoting the absorption of calcium by the gut, but it also influences both the resorption of bone and mineralisation of the matrix. Osteonectin is a kind of non-collagen protein, and is capable of high-affinity binding to both HA and collagen which may facilitate mineralization of type I collagen. Type I bone

collagen is a highly organised substratum onto which bone mineral crystal may deposited. Mineralisation of collagen has been associated with matrix vesicles: originate from osteoblasts and chondrocytes and contain calcium phosphate and assorted enzymes required as requisites for mineralization. Collagen may act as a template for mineral deposition with the process modulated by collagen -phosphorylated sialoproteins, osteopontin, bone sialoprotein, and bone acidic glycoprotein. Osteopontin functions as an inhibitor of mineralisation and meanwhile, osteonectin is the most abundant noncollagen protein and may direct calcium concentration dependent nucleation, and/or stabilise calcium-phosphate(Hollinger and McAllister, 1995). Bone sialoprotein is a nucleator and the role of bone acidic glycoprotein has yet to be determined. Mechanisms of actions and interactions between these noncollagenous proteins and collagen remain to be elucidated.

1.6 Implantation biomaterials

When artificial materials are placed in the body, in terms of the interactions, two points of view may be considered. One is the affect of the physiological environment upon the material, and the other is the affect of the material, as well as its corrosion or degradation products, upon the body fluids and tissues of the surrounding environment. As knowledge has been gained about the complex activities which take place in the human body, the demand for new and improved materials, which can better withstand this highly corrosive environment has greatly increased. The use of ceramic materials for implantation into the human body is presently in the experimental and limited clinical stages. Their obvious chemical and physical properties would initially indicate ceramic materials for surgical implants to be best suited as bone and tooth prostheses.

Biocompatibility: The last two decades have witnessed a growth of interest and significant progress in the research and development of biomaterials. It is now possible to obtain materials that have been designed, constructed, and manufactured for specific clinical and surgical applications. Progress has been made in all categories of materials: metals, ceramics, polymers and composite material systems. However, before a material system is adopted, many important issues such as mechanical performance, biocompatibility, biodegradability, corrosion properties, etc. must be thoroughly evaluated. The fundamental requirements for an implant material include:

1. ***biochemically compatible, non-toxic, non-irritable, non-allergic and non-carcinogenic.***
2. ***biomechanically compatible with surrounding tissue.***
3. ***bio-adhesive in the living tissue.***

Though still today there are no strict standards or regulations, to systematically match the above requirements, except for the toxicity indices, usually there are four levels of standards to be evaluated (Guicheux et al, 1997; Okumura et al, 1997; van Kooten et al, 1997);

Physical tests: Strength and hardness index;

Chemical tests: Corrosion and solubility tests;

Biological tests: In vitro cell or tissue culture and in vivo animal implantation;

Clinical evaluation.

The results of an interaction between the implant material and the living tissue are characterised by different grades of compatibility and resorbability of the implant materials. Materials are graded in the following way.

Incompatible: Release of substances at toxic concentrations, or release of antigens which provoke immune reactions and other reactions such as foreign body reactions, inflammation reactions, necrosis reactions as well as possibly rejection.

Biocompatible: Release of substances in innocuous concentrations. Reaction include encapsulation by fibrous tissue, mild foreign body reaction(giant cells), incorporation into tissue e.g. bone is possible (Occur et al, 1997).

Bioinert: No release of interacting substances, direct apposition of bone possible (Black et al, 1992).

Bioactive: Positive interaction with tissue differentiation, bonding or adhesion to bone along the interface (Lin and Chao 1986).

Inductive: induction of heterotopic bone formation.

Conductive: Serves as a template or scaffold for bone deposition, but only in an osteogenic environment (Silver and Dillon, 1989).

Metals and Alloys: The structure of an implant alloy is on the one hand determined by its chemical composition and on the other hand by the manufacturing process used in the production of the alloy. On the basis of their chemical composition, the clinical implant metals are iron-, titanium- and cobalt- based alloys, such as 313 stainless steel, Ti6Al4V,

and CoCrMo. With a passive oxide film of good adherence at the implant surface, these alloys display good compatibility. Emphases have been stressed with regard to the excellent mechanical properties and workability of the alloys. The theoretical density of titanium alloy (~4.1g/cm^3) is half that of steel (~8.2g/cm^3) and CoCrMo alloy(8.9-9.1g/cm^3) (Exbrayat et al, 1987; Duncheyne and Hasting, 1994; Evans and Benjamin, 1987) approximating that of human cortical bone (2.2g/cm^3). It has excellent mechanical properties, and formation of a TiO_2 film impairs the corrosive action of body fluids. The titanium dioxide layer on the surface of titanium implant leads to the formation of a direct interface with bone tissue, i.e. osseointegration (Ong et al, 1995; Bange et al, 1991; Kothari et al, 1996). Titanium alloy has been applied in the clinic on a large scale. Though the biocompatibility of metals or alloys can not match that of bioceramics, the successful position of substitution of joints by metals or alloys can not be achieved by any other materials (Silver et al, 1994). However, for any metal or alloy, corrosion produced by body fluids can not be avoided. Corrosion or similar chemical degradation caused by the action of body fluid and tissues on the implant not only changes the physical properties of the implant, but, in addition, the products so formed are toxic, causing allergies and/or carcinogenic responses and subsequent isolation and rejection of the foreign body. Aside from the toxicity due to the chemicals, mechanical incompatibility of the implant with the bone and tissue may cause irritation. For example, the difference in the coefficient of the friction between cartilage and the implant in a joint can easily initiate an area of irritation. Another important consideration is the surface condition of the implant material. Differences in crystalline structure, and the presence of surface impurities, may also lead to different responses of the tissues. Hulbert and Young (1969) found that following tissue injury there is a local pH fall to 5.6. A gradual rise ensues with the development on pH close to 7.35 in approximately ten days. From this it is apparent that the implant materials are subjected to a rapidly flowing, highly corrosive environment of changing pH. Pitting corrosion is another form of local attack made by macrophages or monocytes. In general, the degree of tissue reaction is proportional to the amount of constituent released by the implant materials.

Ceramic Materials: Although ceramics are the oldest artificial materials, they were taken into consideration for biomedical applications as the last of the various materials

groups. The systematic and detailed evaluation of ceramics as possible materials for artificial bone and joint replacement devices did not start before the 1960s. At that time metals and plastics had been in use for several decades. Interesting research in the early 1960s showed that the active behaviour of ceramics leads to bonding with the surrounding tissue or to degradation and conversion into bone (Hench, et al, 1971; Hulbert, 1978; Hench and Andersson, 1993). Because of the specific requirement for biomaterials, only some of wide variety of ceramic materials available can be considered for use in implants. Ceramic biomaterials are divided into two groups, depending on the behaviour of the ceramics in the biological environment. One is called bioinert ceramics and the other is called bioactive ceramics (Dubois et al, 1998; Brink et al, 1997). Bioactive ceramics consist of different glasses and calcium phosphate ceramics with a composition and structure similar to the inorganic components of bone. They are characterised by a certain solubility which provokes the surrounding bone or tissue to form a direct bonding to the materials (Kotani et al, 1991). The main restriction for bioactive materials lies in their low strength so they can be used as bulk materials only for low loaded devices (van Blitterswijk,1992). In the bioactive material group, there are the so-called biodegradable ceramics. They are mainly based on calcium phosphate in different modifications. They have high solubility which leads to a gradual degradation and resorption by the surrounding tissue, stimulates the bone to grow on the materials and through its pores, and is believed in some cases to generate a total transformation of the materials into living bone in this way (Heimke and Griss,1980; Vermeiden, 1980). This means that these ceramics could offer new possibilities in reconstructive surgery to fill or bridge bone defects. Therefore, they have stimulated great interest in recent years and their mechanical properties and biological features can be altered by changes in composition and structure. Nevertheless, they seem to be limited in their applicability because of their lack of strength. They can be used only for low-loaded devices and not for long-term implants (Ooishi et al, 1993) .

Bioactive materials for bone substitution may result in a very quick healing reaction without the formation of soft tissue between bone and implant. The connection between bone and implant is strong enough to be resistant to some tensile stress. Whenever tissues are injured or destroyed, the adjacent cells respond to repair them. An immediate response to any injury is the inflammation reaction. If a severely destructive inflammation

process persists and no healing process occurs with 3-5 days, a chronic inflammation process commences and this is marked by the presence of mononuclear cells called macrophages (de Groot and Wolke,1996; Mehlisch et al, 1992). The macrophages are phagocytic and remove debris and bacteria. Generally, the body's reaction to foreign materials is to get rid of them. The foreign materials are extruded from the body if they can be moved or isolated if they cannot be moved. If the material is particulate, then it will be ingested by the giant cells and removed. If the implant is chemically and physically active, a typical tissue response is that leukocytes appear near the implant followed by macrophages, sometimes called foreign body giant cells. If the implant is either a chemical or physical irritation to the surrounding tissue, then inflammation occurs in the implantation site (de Groot and Wolke,1996). The degree of the tissue response to the implant varies according to both physical and chemical nature of the implant. Pure metals (except the noble metals) tend to evoke a severe tissue reaction by oxidation or corrosion. If an oxide layer presents on the surface of the metal, it will exhibit the minimum tissue response reaction, because this oxide layer is a ceramic-like material that is very inert. Ceramic materials, $CaO\text{-}Al_2O_3$, $CaO\text{-}ZrO_2$, $CaO\text{-}TiO_2$, show minimal tissue reaction. However, if the reactivity of the implant material in bone is too high, an inflammation of the surrounding tissue may occur (Ravaglioli and Krajewski, 1992). Inert materials only cause a mild foreign body reaction, which is also typical of autologous material implanted at the wrong place. Inert, in this case, means that there is no provable reaction of the implant materials with the tissue. The normal response of the body is encapsulation of the implant. Antibody reactions of the immune system are based on the presence of protein. As calcium phosphate and other inorganic materials do not carry protein into human body, there is usually not an antibody reaction of the immune system to those materials.

Calcium phosphate ceramic biomaterials: Particular interest has been focused on those ceramics which allow for the formation of some kind of a direct interaction with living tissue, i.e., the bioactive ceramic materials. It has been frequently reported that calcium phosphate ceramics have excellent biocompatibility and bonding with bone for many years (Hulbert, 1978; Park and Lakes, 1992). However, there is still a long way to go until clinical use of calcium phosphate materials is on a large scale. HA clearly confers

an incremental benefit by aiding in the repair of bone defects, supplying additional anchorage and stability at the site of the periodontal pocket, and serving as a barrier for epithelial growth. Bone formation with integration of the material particles occurs from the alveolar ridge to the surface. After 1 year, 2 to 3 mm of new bone is formed around the site of the HA particles (Goodman et al, 1993; Flautre et al, 1996; Nordsletten et al, 1996). However, the friction between cartilage and implant is in certain cases a problem. The coefficient of friction of articulate cartilage is 0.03 to 0.04 while that for any other biomaterial is considerably higher and usually five times higher than the coefficient of friction of bone(Black 1992). Calcium phosphate ceramics are considered to be brittle, of low ductility, and flexural strength. In general, ceramics lack static strength, impact resistance, freedom from notch sensitivity, mechanical reliability and are difficult to fabricate into complicated shapes. The major advantages of ceramics as biomaterials in the muscle-skeletal system are tissue acceptance and tissue adherence.

HA ceramic is a refractory, polycrystalline compound, which has a high elastic modulus (40-117 GPa). Hard tissues such as bone, dentin, and dental enamel are natural composites that contain HA (or a similar mineral) as well as protein, other organic, and water. Enamel is the stiffest hard tissue with an elastic modulus of 74 GPa and it contains the most mineral. Dentin (21 GPa) and compact bone(12-18GPa) contain comparatively less mineral (Torzilli et al, 1981; Park and Lakes, 1992). Historically, calcium phosphate biomaterials have been proposed for utilisation as tooth implants, bone substitutes, e.g. as fillers of bone defects, and resorbable and periodontal bone implants, etc. HA ceramic is an implant material with no significant signs of resorption in animal and human application (de Groot, 1992; de Lange et al, 1992). It has frequently been reported that tricalcium phosphate-$Ca_3(PO_4)_2$ (TCP), which is also confirmed as present in human bone and dentin, is generally regarded as an implant material which is easily integrated into bone tissue resulting in resorption of the ceramic and substitution by living bone (Radin and Ducheyne, 1993; Park and Lakes, 1992). The mechanisms responsible for resorption of TCP ceramic have been the subject of much guess work. It was widely assumed that the chemical solubility of TCP differed greatly from that of HA, thus leading to the postulation that TCP, therefore dissolves much more quickly than HA by a chemical process (Hyakuna et al, 1990;Kotani et al, 1991; Ratin and Ducheyne, 1991). Bone tissues are able to grow into porous biomaterials with pore size over 100um (Nunes et al,

1997; Faucheux et al, 1994; Lugscheider et al, 1994), continuous porosity, i.e., pores with no constrictions. In clinical terms, however, an interconnecting system of pores is necessary because the ceramic block could otherwise become a source of infection, and that could certainly lead to loss of the implant.

Porous HA is used for implantation into bony periodontal defects. Such osseous defects are a major complicating factor in periodontal disease. Different reactions in bony and in soft tissue can be produced by the biocompatible HA ceramics (Eggli et al, 1988; Stuppsi and Ciegler 1992). The degree of giant cell reaction is possibly dependent on the Ca/P ratio or biochemical stability and other factors. Similar to TCP, stoichiometric HA is not stable (Legeros et al, 1993; Radin and Ducheyne, 1994; Ducheyne et al, 1993; Koerten et al, 1999). The implantation of radioactively labelled HA led to the conclusion that there is a substantial dissolution of the material in vivo (Hollander et al, 1991). It is a logical hypothesis that one of the mechanisms underling the phenomenon of bioactivity, i.e., enhancement of bone tissue formation rates and bone tissue bonding, is that dissolution from the ceramic produces a solution which mediated events affecting cellular activity, organic matrix deposition, and/or mineral precipitation. After implantation, the most important reactions take place between calcium phosphate and osteoclasts as well as between calcium phosphate and osteoblasts. In the former case the implant materials are resorbed by osteoclasts, and in the latter case matrix collagen may be produced by osteoblasts on the implant material, constructing the bone bond between implant material and living tissues.

In the process of preparation of calcium phosphate materials, the most important factors are particle size of the powders and the sintering temperature. During sintering, over-sintering may result in shape changes, producing big crystals which result in brittle mechanical properties, and phase changes. It is difficult to obtain pure HA material and usually TCP is present in the sintered materials because of the phase transformation during sintering at elevated temperature. Often HA-TCP biphasic materials are obtained by controlling the parameters of powder synthesis and sintering temperature. In fact, for the bone vacancy filling, more attention has been focused on HA-TCP biphasic biomaterials (Akao, 1981; de Groot, 1990). Chen et al (1994) pointed out that the hard tissue of human beings is composed of a HA-TCP biphasic structure and TCP present in HA biomaterials may improve the biological characteristics, and increase the bioactivity

(LeGeros and Daculsi, 1990). In our research (Ruan and Zhou, 1992), the highly porous material is composed of a HA-TCP biphasic structure. HA and TCP have similar lattice structure, but the latter does not have OH groups and the lattice constants are different from the former. Therefore, they have different mechanical properties. Also, the biological features of HA are different from those of TCP. Compared to HA, TCP is more easily resorbed by osteoclasts (LeGeros and Daculsi, 1990; Van Raemdonck, 1984; de Bruijn, 1994).

In the past 15 years prostheses coated with HA have been developed and animal experiments as well as clinical practice has demonstrated that the time for forming a bone bond between the implant and living tissue may be shortened (Moroni et al, 1993; Oguchi and Hastings, 1994; Hardy et al, 1991). The need for bone cement can be removed if the artificial joints or implants are coated with HA. HA coating is one means of cementless fixation (Schroeder et al, 1988; Couteney et al, 1995; Wolke et al, 1998). Currently, two coating processes are of interest. One of them is called plasma spray, which is the only one commercially available, while the other one, called magnetron sputtering may be used in the near future (de Groot and Wolke, 1996; Anselme et al, 1997; Gaillard et al, 1993). Experiments have revealed that the bonding strength with HA coating on the surface of Ti6Al4V beads is four times higher at 2 weeks when compared with after implantation, three times higher at 4 weeks, and 2 times higher at 6 weeks (Wolke et al, 1998; Bowers, 1992; Soballe and Overgaard, 1998). Normally, the plasma spray technique and flame spray technique are employed to coat HA particles on to the metallic prosthesis and the thickness of the coating layer varied from 20 to 70 μm. However, there are two problems often encountered using the above coating techniques. The problem involves the combination between the metallic matrix and HA particles, and results in lamination and cracking in the coating layer, and the second problem is the phase transition of HA to TCP during coating, because of the higher temperature of over 1200°C required (Gross and Berndt, 1998; Tong, 1997; McPherson et al, 1995). Consequently, the coating layer may be easily resorbed or degraded by osteoclasts and the metallic matrix exposed to the body environment. Some approaches to solve those two problems by controlling the coating conditions or other coating techniques such as magnet tube field spray technique have been attempted(Cottel et al, 1992; Gross and Berndt, 1998; Koerten,1999). However, the further problem is that if the coating film is gradually degraded, then the

substrate materials will be exposed more and more to the surrounding tissue and the interaction will be carried out between substrate materials and tissue directly (Osborn and Newesely, 1980).

Toxic response of tissues and corrosion of biomaterials: When evaluating biomaterials for use as implant materials in human medicine, the reactions that occur in the organism must be known. Implant materials have to be chosen very carefully with respect to chemical composition, to mechanical features, to the response of the organism, to toxic response, and to corrosion behaviour because body liquids are a very aggressive medium. A major cause of failure of a biomaterial in the body is toxicity including inflammation responses, corrosion by the body environment, or attack by the body's immune system (de Groot and Wolke, 1996; Klein et al, 1989; Giridhar et al, 1996; Remes and Williams, 1992). Also, van Luyn (1998) showed that T cells play a pivotal role in the recruitment of macrophages and the formation of giant cells, so the tissue reaction to biomaterials might be modulated by controlling T cell activation. Beside chemical dissolution, cell-mediated effects play an important role: for example, osteoclasts are capable of dissolution of implant materials. Giant cells and macrophage cells incorporate implant materials if degradation of the materials to small grains occurs (Benahmed et al, 1996; de Bruijn et al, 1994; Ziats, 1988). The grains may be as large as 50μm because several cells are able to fuse together for incorporating huge grains (Gomi et al, 1995; Dubois et al, 1998; Ushida et al, 1992). Failure to dissolve the foreign body results in encapsulation of the implant. One of the most important responses of the organism is the ability to dissolve the implant. Factors such as chemical solubility, biological solubility, enzymatic reactions, activity of osteoclasts, or the incorporation of the particles by macrophage cells influence the result (Klein et al, 1989).

The corrosion products prevent mineralisation of newly forming bone. Some macrophages in the adjacent connective tissue contain corrosion products and they may induce a severe foreign body reaction with development of giant cell granulomas (Ziats, 1988; Harada et al, 1996; Evans et al, 1991; van Luyn et al, 1998). This has been observed with amalgam in soft tissue. Another feature which may influence the degree of inflammatory reaction in soft tissue is the shape of the foreign body (Kieswetter et al, 1996). Multi faceted HA particles induce a stronger inflammation than spherical HA

particles (Davies and Maldan, 1997; Nordsletten et al, 1996; Guicheux et al, 1997). There is no doubt that giant cells adjacent to HA and TCP biomaterials, and the presence of a fibrous capsule, are signs of chronic inflammation. This giant cell reaction occurs in every case, but the number of the giant cells varies with the biochemical stability of the materials (de Bruijn et al, 1994; Cook and Mitchell, 1989; Kuboki et al, 1998). A major component of biocompatibility is the toxicity of the biomaterial. Classically, the toxic effects of a chemical are studied at three levels:

1. ***Acute toxicity;***
2. ***Subacute toxicity and;***
3. ***Chronic toxicity***

In the broadest sense, toxicological and adverse effects from the implants may arise from two sources: (1) the leaching of the chemical constituents from the implant materials and (2) material-cellular interactions. A number of consequences which may occur from each of these sources are listed here:

1. ***Leaching of chemical agents from implant material***
   (a) Local tissue response.
   (b) Systemic toxicity
   (c) Allergenic response
   (d) Teratogenic, mutagenic and carcinogenic activities.
2. ***Material-cellular interaction***
   (a) Adsorption of cellular constituents from tissue by the materials leading to changes in the physical, chemical and mechanical properties of the implant materials.
   (b) Adsorption of vital cellular constituents such as antigens, antibodies, hormones, and various circulating drugs.
   (c) Material-blood contact, leading to thrombus formation.
   (d) Material-tissue contact leading to scar tissue and immobilisation of the implant (for example, a breast implant).
   (e) Physical or solid-state carcinogenesis.

Local or systemic toxicity, scar tissue formation as well as immobilisation of the implant could be caused by both type of sources. Most information on the toxicological aspects of biomaterials has been obtained on the chemical agents released from the

materials. The tissue response to injury may vary widely according to the site, species, contamination, etc. However, the inflammation and cellular response for both intentional and accidental injuries are same regardless of the site. Toxicology is the study of harmful effects of a chemical, or biological agent upon animals and humans. The toxic effects of a chemical are studied by in vitro or in vivo methods. This thesis is concerned primarily with the assessment of toxicity by in vitro methods.

1.7 Assessment of toxicity by in vitro methods

Determination of potential cytotoxicity is an important part of biomaterials used in human body. Cytotoxicity assessment is the primary step to screen the biomaterials and is composed of two basic methods in practice: direct contact cytotoxicity evaluation and indirect cytotoxicity evaluation. In the direct contact cytotoxicity evaluation, suitable cells or tissues are cultured on the material specimens and observations including cell adhesion, distribution and migration, proliferation and differentiation are carried out. Adherence of cells onto material surfaces relates to the physicochemical properties of the material surface. Hydrophilic and hydrophobic character and composition of the material's surface have important roles in cell attachment onto the material surface. Basically, a hydrophilic surface is available for cell adhesion mediated by adsorption of proteins from culture medium or by proteins on cell membrane. Improving wettability of the material surface can promote protein attachment onto the material surface, and consequently, cell adhesion at the material surface will be enhanced. However very strong wettability and a very strong hydrophilic surface does not provide a suitable surface for proteins to adsorb onto. For example, polyethylene coated with sulfo-betaine will impair protein adsorption, though the surface shows very strong hydrophilicity, and as a result the adherence of fibroblasts, as well as macrophages, is significantly reduced. The impairing mechanism is still unclear but it is proposed that the very strong combination between the water molecules and the material surface is difficult to break with the result that proteins or cells cannot direct contacted to material surface. Biomaterials are used for contact lenses, artificial blood vessels, and urethra tubes where adherence of proteins and cells to materials surfaces is to be avoided so that tube clotting or blocking or change in optical properties of the contact lens are avoided. Therefore, the

biocompatibility of these biomaterials is not expressed by cell adherence or cell proliferation on the material surface. Beer et al (1997) demonstrated that the plasma deposition of triethylene glycol monoallylether can markedly reduce protein adsorption on plastics.

Osteointegration of biomaterials takes place at the interface between material and tissue. It is a critical point whether or not cells can adhere and proliferate on the materials. During bone integration, cell adhesion is the first important step. The normal activities and death of cells on the materials take place after cell adhesion (Ertal et al 1994). It has been observed that non-adherence can induce apoptosis in endothelial cells. Ertel suggested that cell death due to non-adherence can be called cytotoxicity. Van Koot et al (1997) expanded this point of view as low adhesion is lack of biocompatibility.

Indirect cytotoxicity evaluation is carried out by culturing cells in material extracts, then observing the cell phenotype in the material extracts e.g. by investigating the protein synthesis ability of cells. The toxic materials leaching from materials can influence cell metabolism, resulting in lost of proliferation and differentiation abilities, even in cell death.

To assess cytotoxicity of materials via an in vitro model, selection of suitable cells is also important. Cell culture tests for the assessment of toxicity should where possible use standard cell lines to enable results to be compared between different materials and different laboratory conditions. For testing the toxicity of bone substitutes, osteoclast-like and osteoblast-like cells are suitable because bone substitutes in the human body are mainly attacked by osteoblasts and osteoclasts. Osteoclasts resorb the implant, resulting in biodegradation or loosen of implants. Osteoblasts integrate the implants together with bone tissues.

1.8 Protein adsorption on the implant materials

In order to improve the surface condition of implant biomaterials so that cells may more easily anchor to, and grow on them, a method of surface modification has been developed by immersing the biomaterials in simulated body fluid(SBF) or culture media. Surface modification is a complicated physicochemical and biochemical process, which includes organic reactions and inorganic reactions between the immersed materials and

the immersing media, such as protein adsorption, calcium and PO_4^{-3} release from the material surface into the immersing media, and/or calcium and PO_4^{-3} precipitation onto material surfaces. These events cause changes in the physical microstructure and composition of material surface, and alterations in the components of materials. Therefore, the modified surfaces are available for the attachment, differentiation and proliferation of cells, and also for inhibition of the tissue response to the implant materials.

In inorganic materials research in recent years a central objective of tissue engineering has been focused on the interaction of cells with biomaterial surfaces via protein adsorption onto orthopaedic material surfaces. The reason for doing this is to modify the material surface and enhance cell anchoring, differentiation, and proliferation on the materials, as cell attachment, proliferation, differentiation are thought to be mediated by proteins. The method of protein coating is rather simple. Generally, the biomaterials are immersed in media with protein for up to 28 days and modifications of microstructure, as well as composition, of the material surfaces result in improvement of the biocompatibility of material surfaces. Usually apart from protein deposited onto material surface, some other chemical reactions between the media and the immersed block will take place.

The events of protein adsorption from the media onto the biomaterial surface and their ability to stimulate the interaction between implant and tissue, as well as the nature and degree of the cell or tissue response, depend on the characteristics of the materials, such as its chemical composition (El-Ghannam et al, 1996; Nishizawa et al, 1995), surface condition (Luck et al, 1998; Suzuki et al, 1997; Ong et al, 1998), porosity and density (El-Ghannam et al, 1997), and also the crystal size (Radin and Ducheyne, 1996; Sun, 1999). There are two types of materials used in the clinic which are coated with protein during use: organic polymer biomaterials, for example, polyethylene terephthalate (PET), for haemodialysis membranes, polyetherurethane(PEU), polyetherurethane urea (Brunstedt, 1993), polyurethane, polymethylmethacrylate (PMMA) (Ueda et al, 1995; van Kooten et al, 1997), hydrophilic polyetherester multi-block copolymer to enhance blood compatibility; and inorganic biomaterials, for example, titanium as well as its alloy Ti-6Al-4V (Martin, 1995), silica carbide (Shelton et al, 1996), stainless steel, bioactive glasses (Brink et al, 1997), and bioceramic materials. El-Ghannam et al (1997)

demonstrated that HA adsorbed a wide range of proteins with more extensive adsorption in the range near 30, 80,and 116KD. Anchorage-dependent cells adhere to these materials via adhesive protein adsorbed onto their surfaces. A list of these proteins includes albumin, bone sialoprotein, collagen, fibronectin, osteopontin, thrombospondin, laminin, IgG and vitronectin, etc.

As well as protein adsorption onto the surfaces of materials, physicochemical reactions take place at the solid-liquid interface (El-Ghannam et al, 1997). Taking HA as an example, ions are released from the immersed materials into liquid and in the other direction cations are precipitated onto the material surface, to form bioactive equivalent carbonated apatite(c-HA) (Hench and Wilson, 1984; Thomas, 1997). The bone bonding between implants and living tissue relies on the formation of c-HA. For inorganic implant materials in practice it could be more important to form the bioactive equivalent c-HA than to have protein adsorption onto the material surface. In vitro and in vivo experiments with bone bonding biomaterials have often led to the formation of a biological type of c-HA on the surfaces of these materials (Radin and Ducheyne, 1993). However, protein adsorption on the surfaces of calcium phosphate ceramics usually impedes c-HA formation. Immersion experiments revealed surface transformation reaction to c-HA as the result of dissolution, precipitation, and ion exchange phenomena. The physicochemical reactions of dissolution and precipitation driven by solid-solution equilibrium are important mechanisms for bone integration of materials.

Protein adsorption onto polymeric substrates has two functions. In some cases, protein adsorption enhances cell attachment onto the substrates and promotes cell differentiation and proliferation. The RGD and the ligands on osteoblast cell surface play a significant role in protein mediated osteoblast attachment (Thomas et al, 1997; Stubbs et al, 1997; Dee et al, 1998). Not only osteoblasts, but almost binding of all of the anchorage-dependent cells to substratum is mediated by extracellular proteins and/or proteins adsorbed onto substratum. All of these proteins have the RGD sequence as the minimal core active site in one of their adhesive domains. RGD tripeptides are recognised by cell surface receptors, and mediate interactions between cells and material surface. (Ruoslahti and Pierschbacher, 1986). The binding mechanisms of RGD-ligands of a few proteins, such as fibronectin and vitronectin have been widely investigated, but there is

along way to go to understand the mechanisms and consequences of protein adsorption. The vitronectin protein receptor plays an important role in promoting cell attachment on bioactive glass (Ruoslahti and Pierschbacher, 1986; Thomas et al, 1997; Dee et al, 1998). Collagen added in serum is useful for calcium-deficient HA formation in tissue culture media and in deionised water (Deyme et al, 1986; Baszkin and Boissonnade, 1993). Evidence from experimental studies indicates that the selective adsorption of fibronectin on the surfaces enhances cell adhesion and osteoblast phenotypic expression. Adsorption of serum protein can affect the local contacts (Martin and Brown 1994) and the cytoskeletal arrangement of cells which subsequently affects cell interaction (Howlett et al, 1994; El-Grannam et al, 1995). The cytoskeleton receives signals from the extracellular matrix, controlling cell function, differentiation and proliferation. Vitronectin is required for bone-derived cell attachment, spreading and spatial distribution when exposed to substrates (Thomas et al, 1997; Howlet et al, 1994). Serum depleted of vitronectin resulted in greatly reduced levels of cell attachment, spreading and spatial distribution on quartz glass and HA. Albumin adsorption generally inhibits adhesion, but can promote cell attachment under certain conditions. For example, albumin adsorption may cause the conformation or density of vitronectin to be optimal for cell attachment (Baszkin and Boissonnde, 1993; Brink et al, 1997). Co-adsorption of low concentrations of albumin (2~5ug/ml) with fibronectin causes conformation changes to the adsorbed fibronectin resulting in active cell spreading. The adsorption behaviour of albumin on hydrophobic surfaces is extremely rapid, attaining saturation surface concentration within a few minutes. However, the adsorption behaviour of albumin on hydrophilic surfaces is slower. Experimental data revealed that albumin adsorbs strongly to polyethylene surfaces and can not be eluted with the buffer solution (Lück et al, 1998; Taborellim et al, 1995). This would indicate that albumin molecules undergo significant conformation changes leading most probably to a surface denaturation.

Spatial distribution of cells is dependent not only on the surface characteristics but also on the media conditions(i.e. presence of serum protein) used during experiments. Cell function is generally mediated through either adsorbed protein or immobilised peptides on materials(especially on polymer materials). The surface chemistry and physical morphology of a material dictates the characteristics of the adsorbed protein layer, which then determines the cellular response to that material. It has been established

that adsorbed proteins interact with molecules on the cell membrane, such as integrins. These interactions with integrins usually occur at the RGD peptide sequence which presents on a number of extracellular matrix proteins, such as albumin, fibronectin, vitronectin, collagen and bone sialoprotein. Steele et al (1995) reported that vitronectin was the major adhesive protein in serum responsible for cell attachment. In vitro, .fibronectin is the primary protein leading to adhesion of fibroblasts and bone-derived cells to synthetic polymer surfaces (Agathopoulos and Nikolopoulos, 1995; El-Ghannam et al, 1997; Frank et al,1990,; Hynes and Yamuda, 1982). Compared with the research on protein adsorption onto polymer biomaterials, less work has been carried out on protein adsorption onto bioceramic materials. Protein adsorption onto bioactive ceramic materials is greatly dependent on the composition and on the crystallised conditions (Suzuki et al, 1997). It is likely that adsorbed proteins prevent degradation of the original crystal, which is required for formation of new phases via solution-mediated physicochemical reactions. The lag time to begin dissolution of the original crystallites in the absence of serum, was delayed in the presence of serum proteins (Ha et al, 1996; Martin and Brown, 1994). Though proteins adsorbed onto material surface may impair the formation of new phases, they promote osteoblast attachment onto the material surface, and consequently, promote cell proliferation and secretion of the extracellular matrix.

1.9 The aims of the research project

In cases involving large defects created by tumour resection, trauma, and skeletal abnormalities, it is unavoidable to use orthopaedic materials to repair the damaged bone, and to help recovery of bone physiological function and mechanical properties. Up till now, there are no suitable materials to perfectly match the physiological and mechanical requirements of natural bone. Metal, ceramic, or polymer materials have been considered, and although they can partially satisfy most requirements, they do not meet the whole requirement. Today, there is more research to improve the mechanical properties of the calcium phosphate ceramic biomaterials, and to investigation the biocompatibility of the biomaterials with different ratio of Ca/P. In this project the in vitro response cells to the materials will be accurately investigated. Based on social and moral altitudes, and cost,

considerable experiments are carried on the characteristics of the materials in vitro rather than in vivo.

Aims of the research

The aims of the present research are:

To process and characterise the HA biomaterials and HA-Spinel composite biomaterials by use of simple laboratory equipment and facilities.

To evaluate the biocompatibility of dense and porous calcium phosphate biomaterials in a cell culture model.

To reveal the interfacial reaction between ceramic biomaterials and tissues via an vitro model.

Our experiments will improve understanding of the responses of osteoblasts to both dense and porous calcium phosphate materials. They will involve measurement of the distribution of osteoblasts on the materials, and growth of the osteoblasts on the materials, evaluation of collagen synthesis function of osteoblasts on the calcium phosphate and the observation of protein adsorption on the calcium phosphate materials.

Chapter 2 Synthesis of Materials

2.1 Introduction

The modern era of calcium phosphate-based biomaterials started 30 years ago (Levitt et al, 1969) with the first publication in the then newly founded Journal of Biomedical Materials Research. Since then, calcium phosphate biomaterials have gained a distinct place in the biomaterials research field, as can be judged by the large number of publications and presentations.

Depending on the Ca/P ratio, calcium phosphate biomaterials mainly include HA with Ca/P 1.67, β-TCP with Ca/P 1.50 and α-TCP with Ca/P 1.50. I selected the apatite form of calcium phosphate since it is regarded to be most closely related to the mineral phase of bone and teeth. Table **2.1** gives the average composition of minerals in specimens of human calcified tissues.

Table 2.1. Average composition of mineral in specimens of human calcified tissues as far as the major components are concerned(van Raemdonck et al, 1984; Ravaglioli and Krajewski, 1992).

Component	Bone % wt.	Dentine % wt.	Enamel % wt.
Ca	36.7	36.8	36.4
P	16.0	18.0	17.1
Na	0.77	0.38	0.64
Mg	0.46	1.24	0.43
F	0.04	0.03	0.01
Ca/P	1.77	1.58	1.64

$Ca_{10}(PO_4)_6(OH)_2$ (HA) has the Ca/P atomic ratio of 1.67 and theoretical density of 3.15 g/cm^3. $Ca_{10}(PO_4)_6(OH)_2$, crystallised into hexagonal rhombic prisms, is the main constituent of hard tissues such as bone, dentin, and enamel. The first crystallographic analysis was carried out by Posner et al (1958). The atomic structure of HA projected the c axis onto the basal plane is given in Figure **2.1**. Six of the ten calcium ions in the cell unit are associated with the hydroxyls in the columns, resulting in strong interactions between them. The ideal Ca/P ratio of hydroxyapatite is 10:6 and the calculated density is 3.15g/cm^3. However, apatite forms a family of solids with general chemical formula

$M_{10}^{2+}(XO_4)_6^{3-}Z_2^-$, where M, X and Z refer to metal ions, oxides, and non-metallic ions respectively. These apatites are frequently nonstoichiometric: 1 mole of apatite may contain less than 10 moles of metallic ions at the M^{2+} position, and less than 2 moles of anions at Z^- position. However, the number of moles at the XO_4^{3-} position always remains 6. The M^{2+} ions are double charged cations like Sr^{2+}, Ba^{2+}, Pb^{2+}. The XO_4^{3-} ions are anions such as CrO_4^{3-}, VO_4^{3-}, while the monovalent Z^- ions are F^-, OH^-, C_2^-, and HCO_3^- ions (van Raemdonck et al; 1984, Muster, 1992). The substitution of OH^- with F^- will give great chemical stability due to the closer co-ordination of F^- by symmetric shape as compared to the nonsymmetric hydroxyl of two atoms. A small percentage of F^- ions greatly increases the acid resistance.

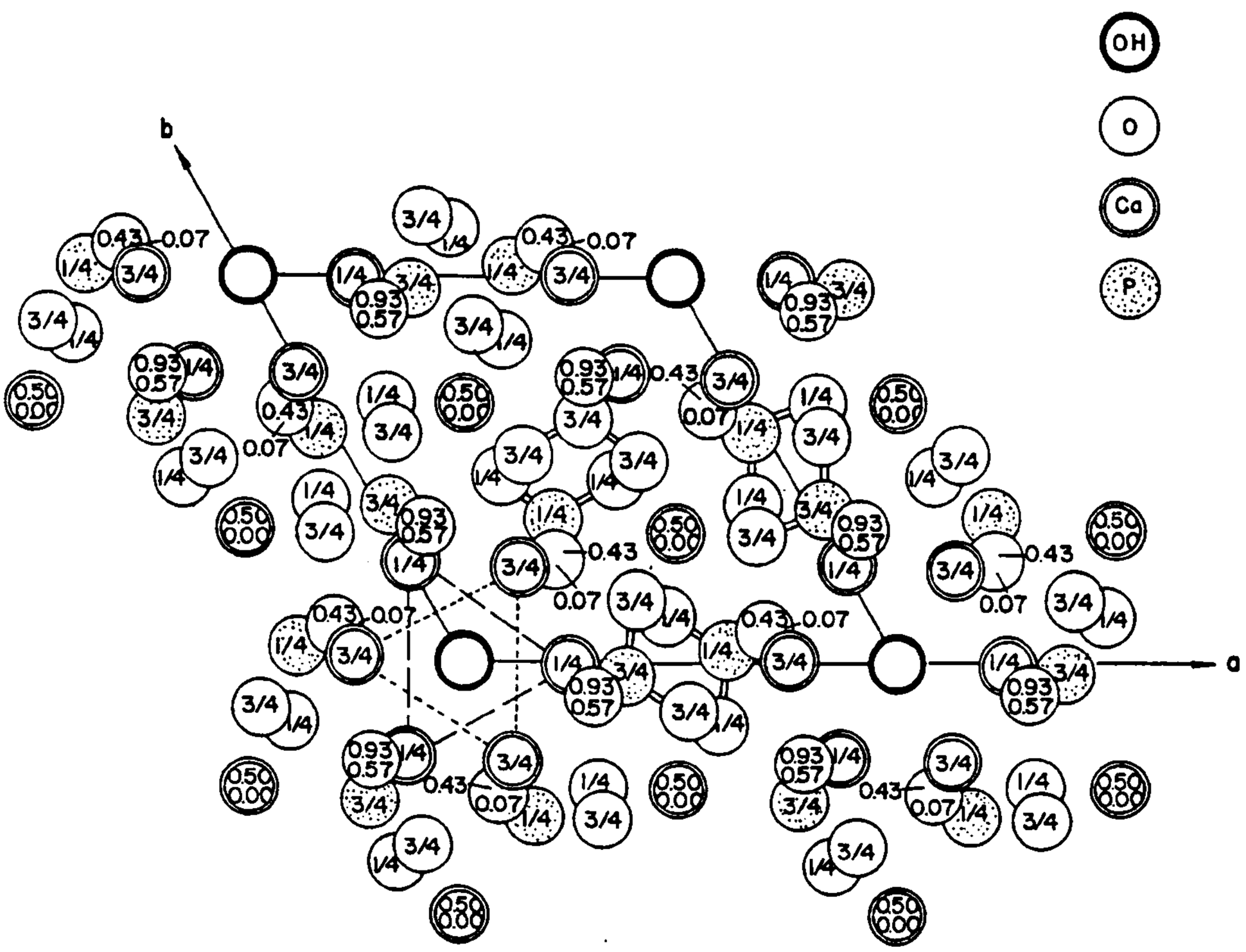


Figure 2.1. Hydroxyapatite (HA) structure projected down the c axis onto the basal plane (from Park and Lakes, 1992).

There is a wide variation in the mechanical properties of synthetic calcium phosphates, which is the result of variation in the structure and in manufacturing processes. Depending on the final firing (sintering) conditions, the calcium phosphate can be HA or TCP, tetracalcium phosphate monoxide and biphase calcium phosphate (HA-TCP) because in many cases both types of structure exist in the same final product. Polycrystalline HA has a high elastic modulus of 40-117 GPa (Park and Lakes, 1992). Compact bone has an elastic modulus of 12-18 GPa, and dentin 21 GPa(Park and Lakes, 1992; Ravaglioli and Krajewski, 1992). Among the most interesting properties of HA and TCP as biomaterials are their excellent biocompatibility.

Indeed they can form a direct biochemical bond with hard tissues. In my experimental trial (Raun et al, 1993b; Wen et al 1993), new lamillar cancellous bone was formed around implanted dense and porous HA materials during animal tests and clinical application. The rate of bonding of bioactive implants to bone depends on the composition of the material. For most implants, failure originates at the interface between the biomaterials and its host tissue. Two factors, poor biomechanical properties and poor biochemical properties contribute to this interfacial failure. Many types of implants for repair of the skeletal system are in contact with both trabecular and cortical bone. It seems to be impossible for most implant materials to produce similar gradients of stiffness between an implant and its host tissue. However, one method of simultaneously improving both the physical and chemical properties of the materials is to produce a composite material combining excellent biochemical and mechanical properties. Therefore, the composite implant materials of metal or alloy coated with bioceramics and ceramic-bioactive glass composites have been developed rapidly during the past few years(Knowles et al, 1995; Suwa et al, 1995; Hayashi et al 1990; Gross et al 1998).

Continuing advances in the field of implantable calcium phosphate biomaterials have produced impressive results concerning their biocompatibility and their ability to stimulate tissue formation. The nature and the degree of the tissue response depends on the characteristics of the material such as its chemical composition, surface texture, porosity and density, shape and size. Host tissue responses to the biomaterials are generally assessed by morphological and histological examinations of the implant site in order to evaluate their biocompatibility with respect to cytotoxicity. On implantation, numerous cell populations and chemical factors are concerned in the tissue response, and

the in vivo experiments do not allow the examination of the reactions of a specific cell to the substrate. In order to determine the sequences of the events and the parameters influencing the interactive process, culture of cells in the presence of biomaterials is of great value. The responses of specific cell populations at the interface of the implanted materials determine biocompatibility of implants.

Many different methods have been developed to make precipitates of calcium phosphate powder materials, bulk materials and porous materials(Jarcho et al, 1976; Doremus, 1983, Akao et al, 1981). In addition to the regular scientific literature, many patents have been filed. In 1987 alone there were 126, their object being the manufacture of prostheses or prosthetic materials with properties like those of natural tissue(de Groot et al, 1990). Dense ceramics are usually made by compressing a powder into a pellet, which thereafter is subject to heat treatment that causes the powder particles to fuse together by means of solid-state diffusion or liquid state sintering. Depending on variables such as sintering temperature, sintering time, and particle size, a dense shape can thus be produced. Calcium phosphate compound powder can be easily prepared in the laboratory: mixing ammonium phosphate and calcium nitrate results in a precipitate, which results in the composition of stoichiometric HA, if variables such as pH and various ionic concentration are well chosen. Several methods are used to introduce macropores into a bioceramic material. Holes can be drilled into the fired body, but a more appropriate technique is based on mixing the starting powder with appropriately sized organic powders, threads, or sponges. The organic additives burn out and leave behind their replicas as voids when the green body is heated up to sintering temperature. Porous implants allow tissue ingrowth. The ingrowth is considered desirable in many contexts, since it allows a relatively permanent anchorage of the implant to the surrounding tissue.

Design of implants is also a very important area of consideration in the fabrication of implants. Sharp corners, edges, and crevices must be avoided since these areas act as centres of stress concentration and localised corrosion, or other types of chemical degradation, particularly when subjected to large alternating and repeated stresses in the highly corrosive body fluid. Another important consideration is the surface condition of the implant materials. In general, the more homogeneous the surface the more resistant it will be to disintegration (Gross and Müller-Maic, 1990; Wilke et al, 1989). The

similarities between bioceramics and bone extend to their mechanical properties, which include comparable specific gravity, comparable coefficients of friction and comparable strength properties(Park and Lakes, 1992). In clinical practice, the artificial grooves and pores on the implant are available for tissue ingrowth, hence the integration time is shortened.

In the present study, we set out to prepare suitable calcium phosphate biomaterials with a composition and microstructure close to that of the calcium phosphate biomaterial that has successfully been used in the clinic. The aim was to investigate the basic physical and mechanical properties as well as to reveal the microstructure.

2.2 Materials and Methods

2.2.1 Material processing

Both HA ($Ca_{10}(PO_4)_6(OH)_2$) and Spinel ($MgAl_2O_4$) powders used in this research were synthesised by chemical precipitation. The reactions taking place during precipitation are as follows:

$$\mathbf{5Ca(NO_3)_2(0.415\ M)+3(NH_4)_3PO_4(0.25\ M)+NH_4OH=Ca_5(PO_4)_3(OH)+10NH_4NO_3}$$

$$\mathbf{MgCl_2(0.25\ M)+2AlCl_3(O.5\ M)+8NH_4OH=MgAl_2O_4+8NH_4Cl+4H_2O}$$

For the precipitation of $MgAl_2O_4$, the reaction solution was maintained at 80°C, and for the precipitation of HA, the reaction solution was maintained at 60-65°C. Meanwhile, the reaction solutions must be continuously gently stirred in order to favour the reaction. Precipitates were washed with distilled water and the water was filtered with a funnel. Then the powders were crystallised at 800°C, for 4hr. To ensure that the powders have higher sintering activity both HA and Spinel powders were milled in an alumina circular mill for 2 hours and 36 hours respectively. As a consequence of this processing, HA powder had mean particle size of ~0.9μm(BET analysis: Thümmler and Oberacker, 1993), and $MgAl_2O_4$ (Spinel) powder had a mean particle size of ~1.4μm(BET analysis). For fabrication of dense HA-Spinel(HS), HA powder and Spinel powder were mixed in an alumina cylinder containing ethanol for 2 hr, and dried at 100°C over night. Due to lack of plasticity in the ceramic particles at room temperature, 0.5% (w/w) of polyvinyl alcohol was added as an organic binder and mixed with the powders, to help to bind the powder together. Powder compacting was carried out in a steel die on an Instron, with compact density being 55% of theoretical density. The HA and HA-Spinel green compacts were sintered at 1100°C and 1450°C respectively in air, for 4 hr. After sintering the dense HA materials had a relative density of over 95%, 3.0g/cm^3, and the dense HS material had a relative density over 93%, of 3.10g /cm^3, compared to the theoretical density of HA (3.15 g/cm^3) and HA75%-Spinel25% (3.28 g/cm^3) respectively.

By changing the compacting pressure and sintering temperature, HA and HA-Spinel materials with different porosity could be obtained. The porous HA, and the HA-Spinel materials with relative density about 75-80% theoretical density were compacted

at half the pressure used to compact the dense materials and sintered at 1000°C for the HA and 1300°C for the HA-Spinel materials. In order to fabricate the highly porous HA material, the HA powder was mixed with polyvinyl alcohol powder and the solution composition is described as follows:

100g HA powder + 150ml water + 5g Polyvinyl alcohol powder

In this way a HA slurry was obtained, and then the slurry was used to soak a polyethylene sponge which had pore size between 300 to 500 μm. The slurry was dried at 37°C for 48 hr, followed by sintering in a furnace at 1250°C in air, for 4hr. Flow chart of the preparation of dense and porous calcium phosphate based biomaterial is shown in Figure **2.2** and the sintering procedures for both dense and porous materials are shown in Figure **2.3**.

2.2.2 Microstructure analysis

Light microscopy and scanning electron microscopy(SEM)(Jeol JSM 840A SEM) were use to observe the morphology of the HA and TCP powder, to investigate the microstructure of sintered materials, and to measure the pore size and pore size distribution of the porous HA material on an area of 36mm^2 at the centre of the specimens. X-ray diffractograms (Sieffert XRC-2 Diffractometer) were recorded to investigate the phase structure of the sintered materials to judge the phase transformation in the sintered materials. Transmission electron microscopy(TEM) (Hitachi S-450 TEM) was used to investigate the crystal structure and atom lattice of the sintered materials.

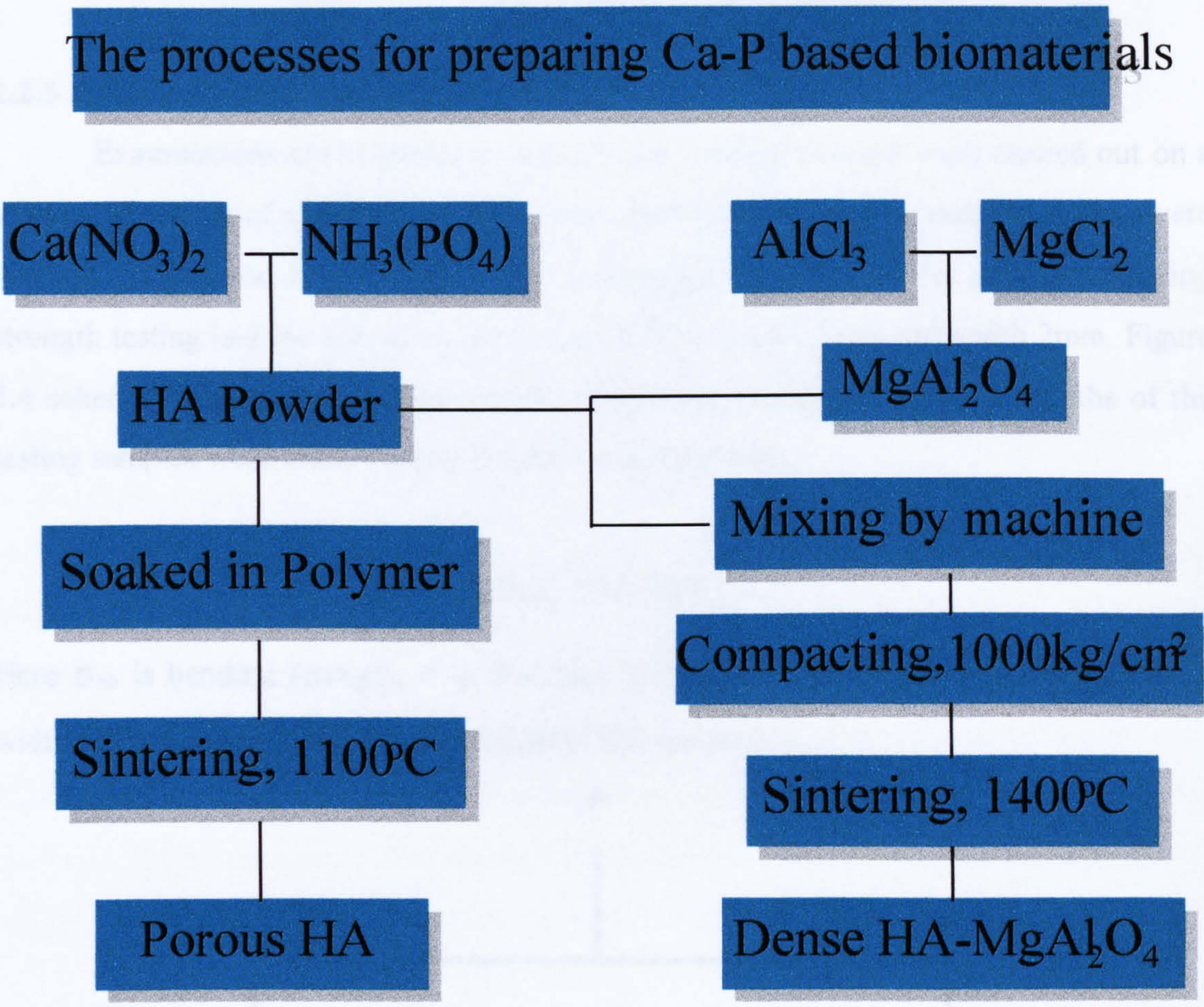


Figure 2.2. Preparation processes for dense HA-Spinel and porous HA biomaterials.

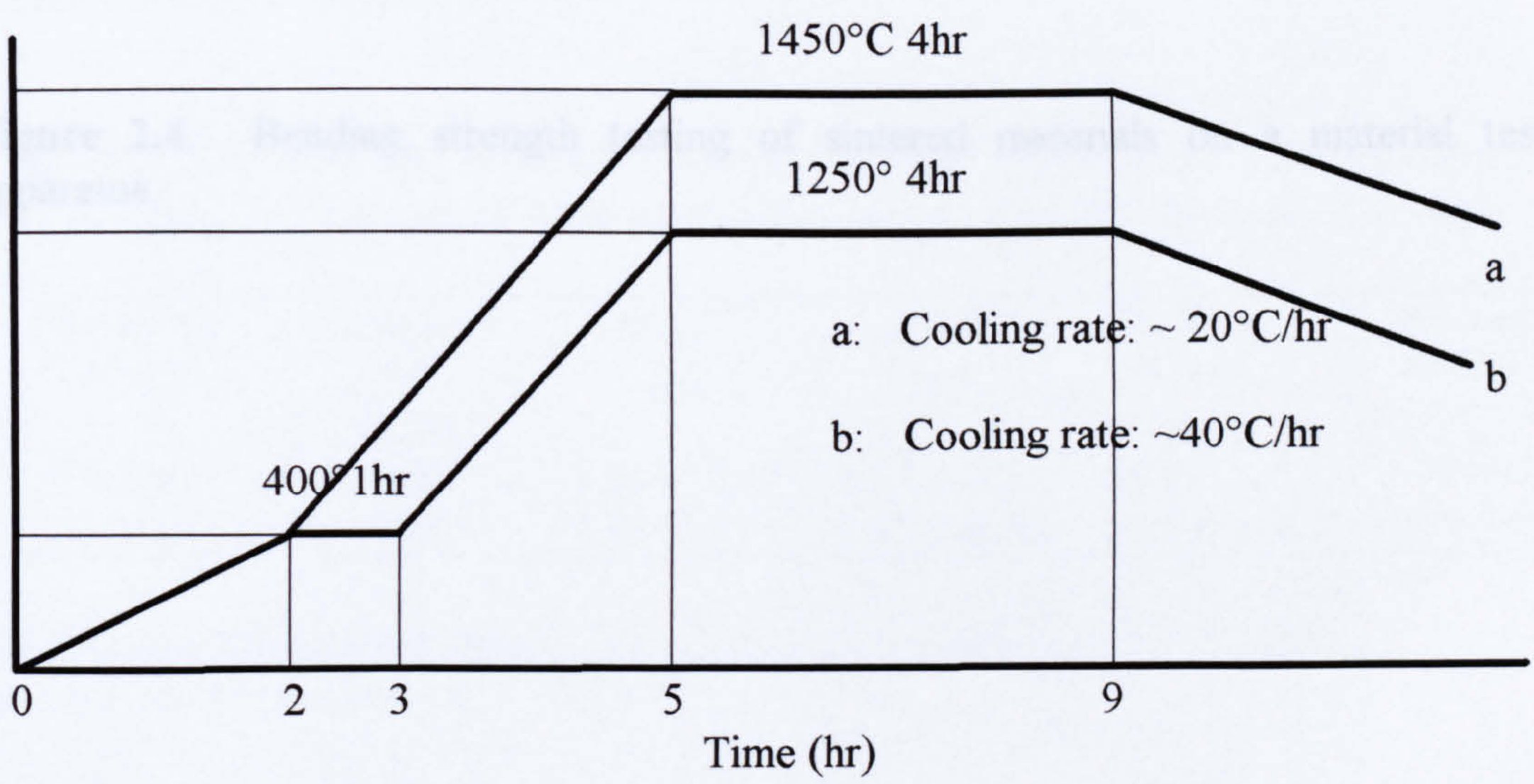


Figure 2.3. Sintering processes and parameters for making dense HA-Spinel and porous HA biomaterials.

2.2.3 Examination of mechanical properties

Examinations of compressive strength and bending strength were carried out on a Instron. The sizes of cylinder specimen to be used for compressive strength testing were diameter 10mm and height 10mm. The rectangular specimen to be used for bending strength testing had the following size length 35mm, height 3mm and width 2mm. Figure **2.4** schematically show the measurement of bending strength. Bending strengths of the testing samples were calculated by the following equation;

$$\sigma_{bb} = 3LP/2bh^2$$

Here σ_{bb} is bending strength, P is the load, L is the span between two supports, b is width of the specimen, and h is the height of the specimen.

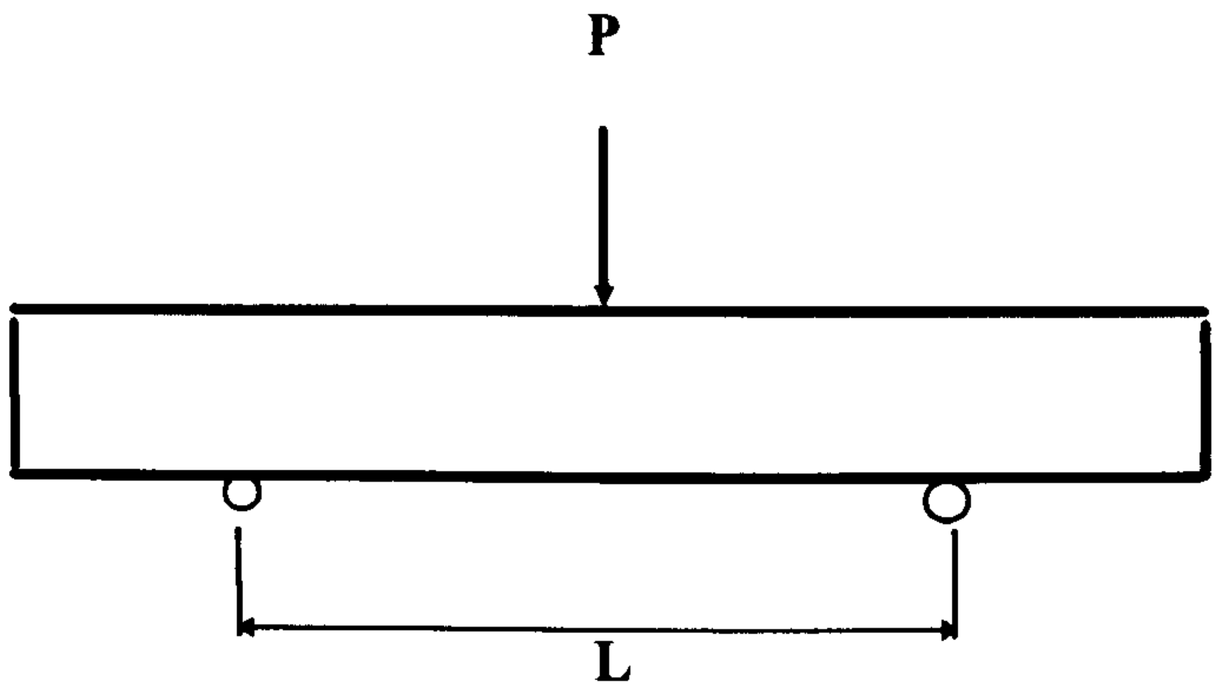


Figure 2.4. Bending strength testing of sintered materials on a material testing apparatus.

2.3. Results

2.3.1 Material physical and mechanical characteristics

X-ray diffraction patterns of dense HA sintered at 1250° and dense HA-Spinel sintered at 1450°C are shown in Figure **2.5** and **2.6**. According to the X-ray diffraction spectrum, there were no HA•Spinel compounds or TCP•Spinel compounds present in sintered materials. X-ray diffraction also revealed that after sintering at elevated temperature, there was some TCP present in the sintered HA material and the HA-Spinel material. Therefore, the porous HA material is composed of crystalline HA and crystalline TCP and the dense HA-Spinel is composed of crystalline HA, TCP, and crystalline $MgAl_2O_4$. Because of the different thermal expansion coefficients which result from volume change when HA is transformed to TCP, microcracks occasionally were found in the dense sintered HA material. By controlling the conditions of powder synthesis, the amount of TCP in sintered materials could be decreased to less than 5% in weight, but it is quite difficult to obtain pure HA material after sintering.

The volume change and mass change of green porous HA pellet and sintered porous HA materials, before and after sintering are given in Table **2.2**. Obviously the pore size was dominated by the pore size of the polyethylene sponge. The data show that the larger the pore size of the sponge, the more powder slurry filled in, and the higher the product density after sintering.

Table 2.2. The mass and volume variations of polyethylene foam materials before and after sintering. Data are given as Mean+/-Stdev (n=18). *P<0.05 by Student's t-test.

Sponge size (mm)	**Mean pore size** (μm)	**Mass**$^{(1)}$ (g)	**Mass**$^{(2)}$ (g)	**Shrinkage** (vol.%)	**Product density** (relative %)
32x20x11	500	7.30+/-0.47	2.54+/-0.34	54.5+/-6.7	~75+/-7
32x20x11	300	7.70+/-0.61	2.97+/-0.35	54.5+/-6.7	~55+/-4*

(1) before sintering treatment,
(2) after sintering treatment;

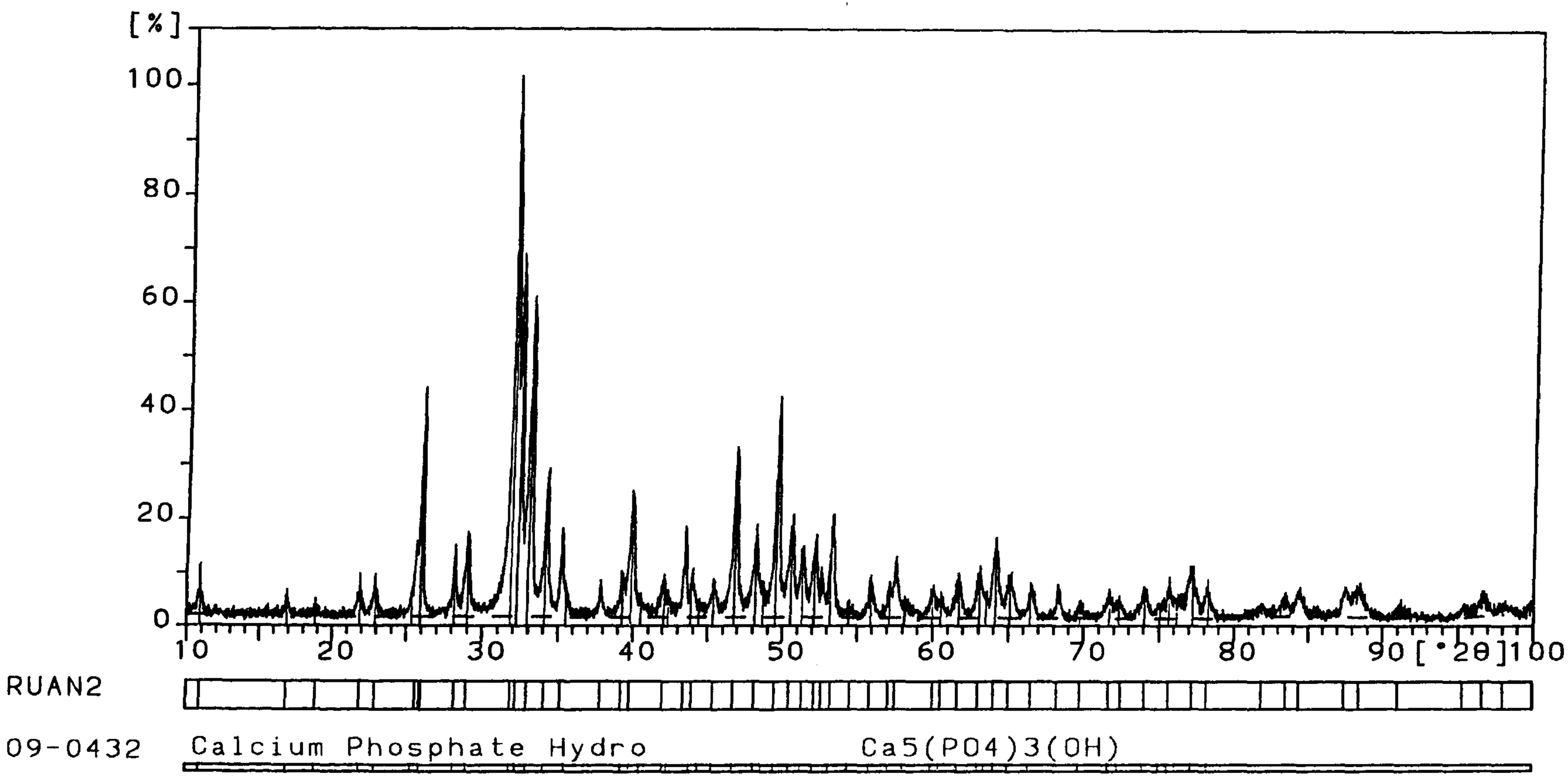


Figure 2.5. X-ray diffraction patterns of dense HA biomaterial after sintering at 1250°C, 4 hours. The sintered material was composed mainly by HA and a little TCP crystals and showed that there was not significant phase transformation during sintering at 1250°C. The patterns show that the material was well crystallised. (horizontal axis: diffraction angle ($2(\theta)$).

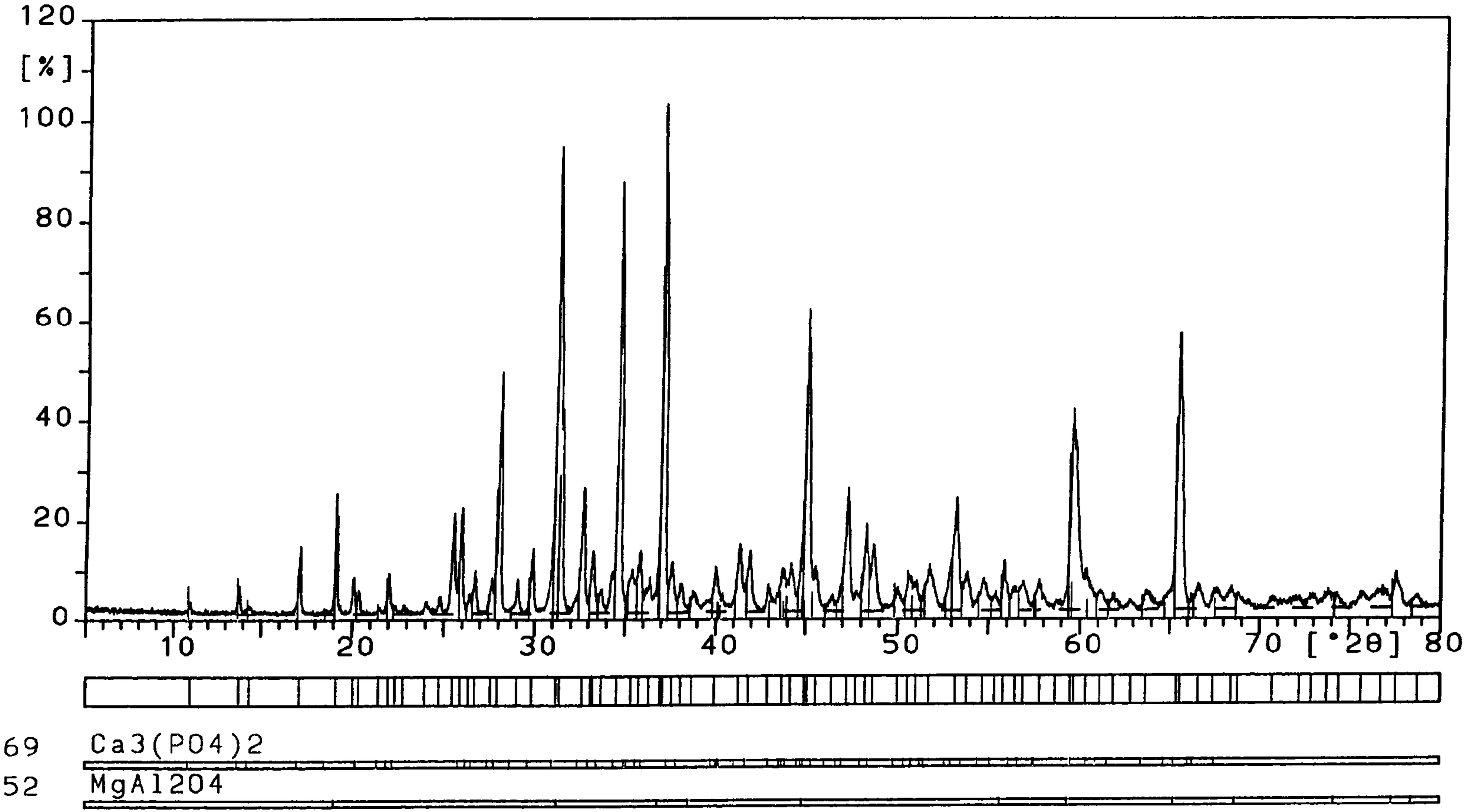


Figure 2.6. X-ray diffraction patterns of dense HA-Spinel after sintering at 1450°C, 4 hours. The sintered material was composed by HA, TCP and $MgAl_2O_4$ crystals and showed that there was significant phase transformation from HA crystalline to TCP crystalline during sintering at 1450°C. The patterns show that the material was well crystallised. (horizontal axis: diffraction angle (2(θ)).

Partial mechanical properties of sintered dense HA and dense HA-Spinel materials are given in Table **2.3**, and Table **2.4**. The mechanical properties were extremely affected by sintering temperature.

Table 2.3. Mechanical properties of sintered HA materials. Results are given as Mean+/-Stdev (n=9). *P<0.05 compared with samples sintered at 1100°C by ANOVA followed by Dunnett's test.

Sintering temperature (°C)	**Sintering density** (relative density, %)	**Compressive strength** (MPa)	**Bending strength** (MPa)
1050	93+/-2.0	174.12+/-24.33*	73.50+/-20.09
1100	96+/-1.7	289.77+/-55.31	82.32+/-24.95
1150	96+/-1.4	201.12+/-38.45*	80.36+/-23.82
1200	96+/-1.4	211.7+/-46.48	61.76+/-17.83

Table 2.4. Mechanical properties of sintered HA-Spinel materials. Results are Mean+/-Stdev (n=9). *P<0.05 compared with samples sintered at 1350°C by Student's t-test.

Sintering temperature (°C)	**Sintering density** (relative density, %)	**Compressive strength** (MPa)	**Bending strength** (MPa)
1350	90+/-1.7	254+/-67	148+/-37
1450	93+/-1.3*	290+/-92	154+/-42

2.3.2 Microstructure analysis by light microscopy, SEM and TEM.

Metallographic analyses of sintered dense HA and HA-Spinel were shown in Photograph **2.1**. As the relative density of the sintered products was over 92%, there were no macropores present in the materials. Compared with metal or alloy metallurgical graphs, it is difficult to observe the details of the crystal morphology by optical microscopy. HA powder morphology and Spinel powder morphology are shown in Photograph **2.2**. HA powders have a complicated structure, and the powders are assembled from fine crystals, which means that HA powder has a large surface area and a higher driving force for sintering. Spinel powders have a smoother surface structure.

Photograph **2.3** is the SEM observations of the dense HA-Spinel material and the porous HA Material. Some isolated pores were present in the HA-Spinel materials and the pore size was less than 3 μm. Most of the pores in the porous HA material were over 200 μm in size and the pores were interconnected with each other. The pore size distribution in the porous HA material (mean pore size 315 μm) is shown in Figure **2.7**. The investigation of crystalline structure in sintered dense HA-Spinel materials is shown in Photograph **2.4**. There were three crystals in Photograph **2.4** (A), two being HA and one being Spinel. HA and Spinel combined together by crystal boundary. The crystallographical analysis of HA is shown in Photograph **2.4**(B), and it shows a typical hexagonal diffraction pattern.

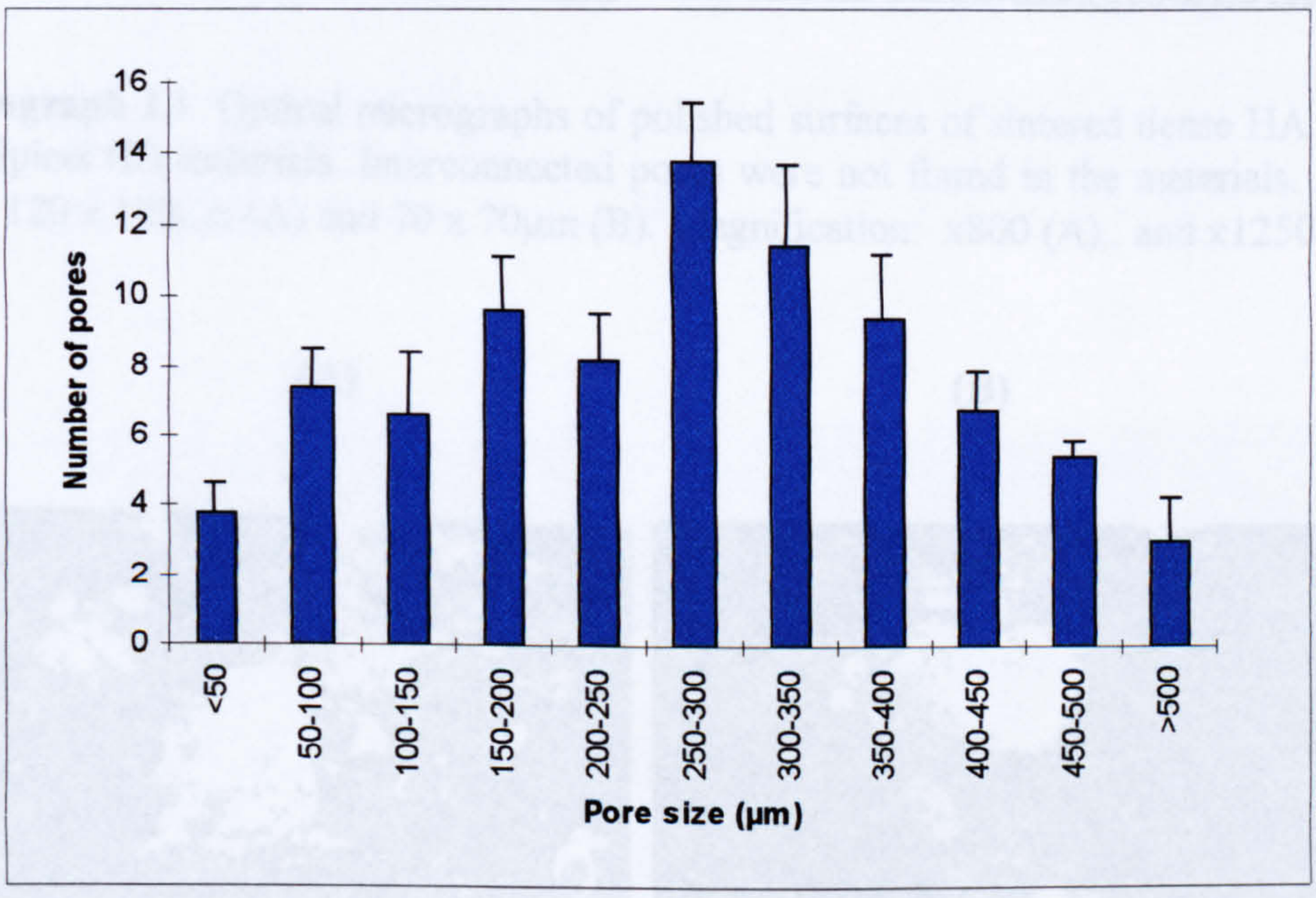
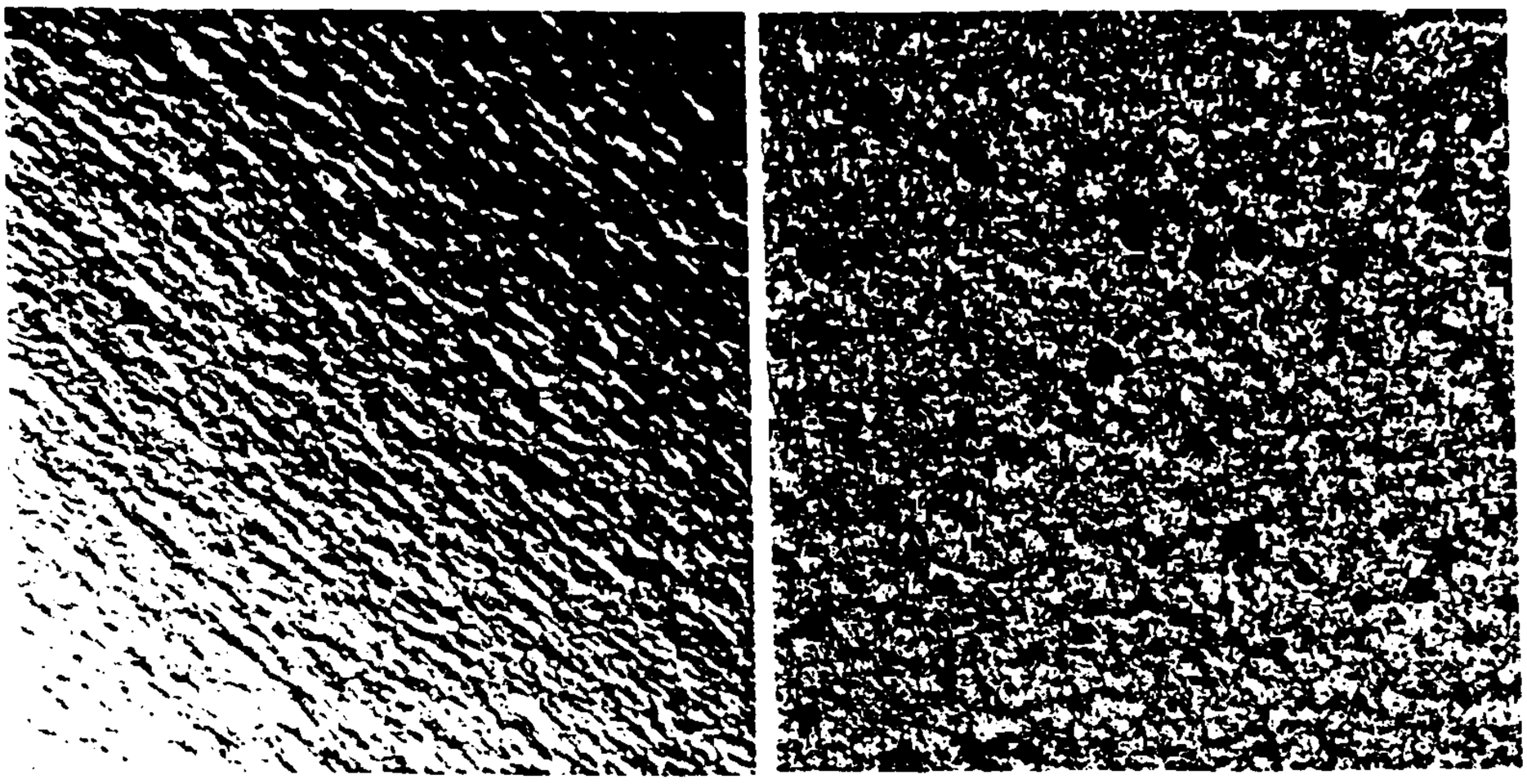


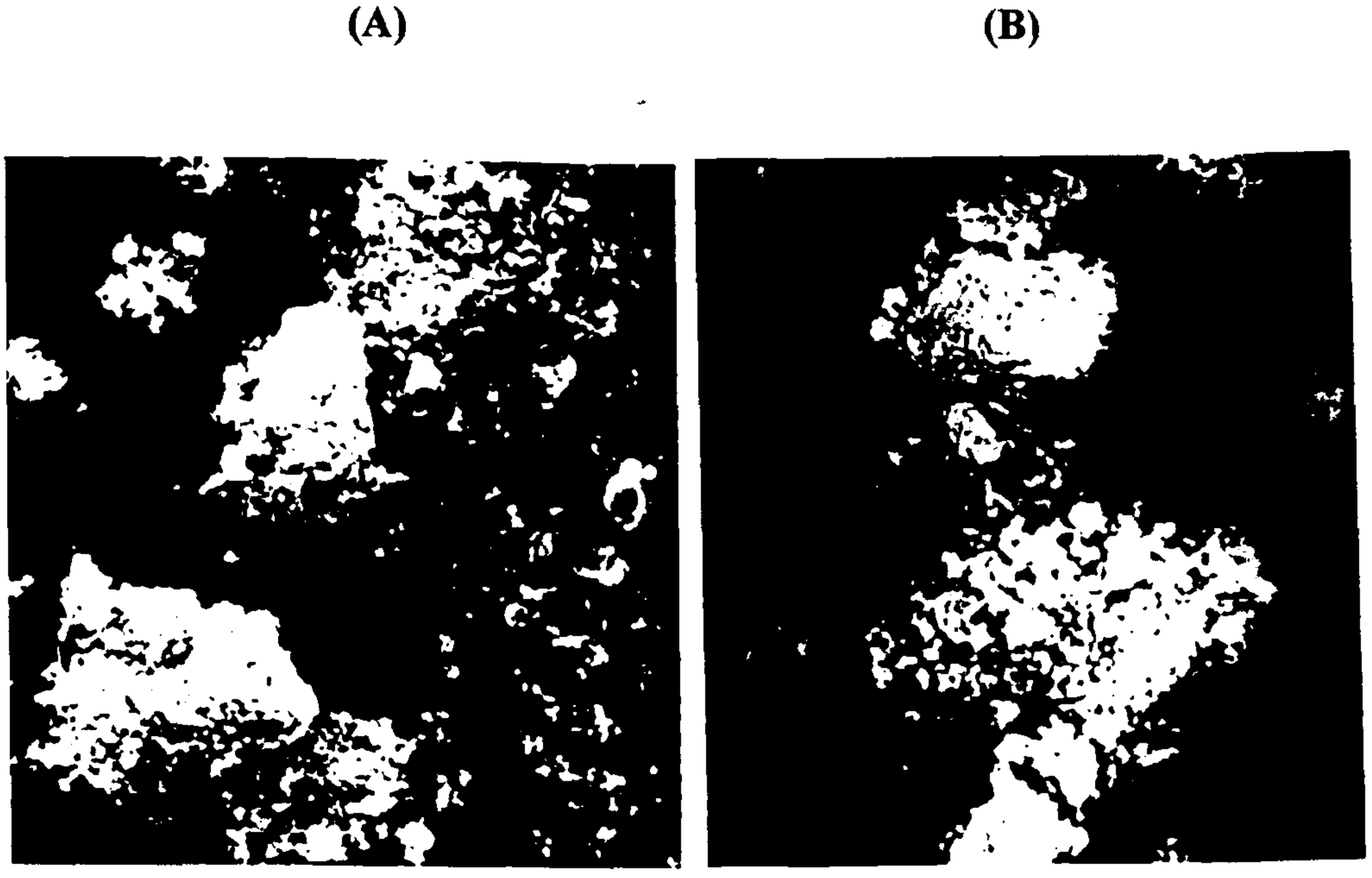
Figure 2.7. Pore size distribution in the porous HA material with mean pore size 315μm. All data were based on 5 samples, and 400 pores were measured on the polished surface at the specimen centre. Each imaging area: 6mm x 6mm.

(A) (B)

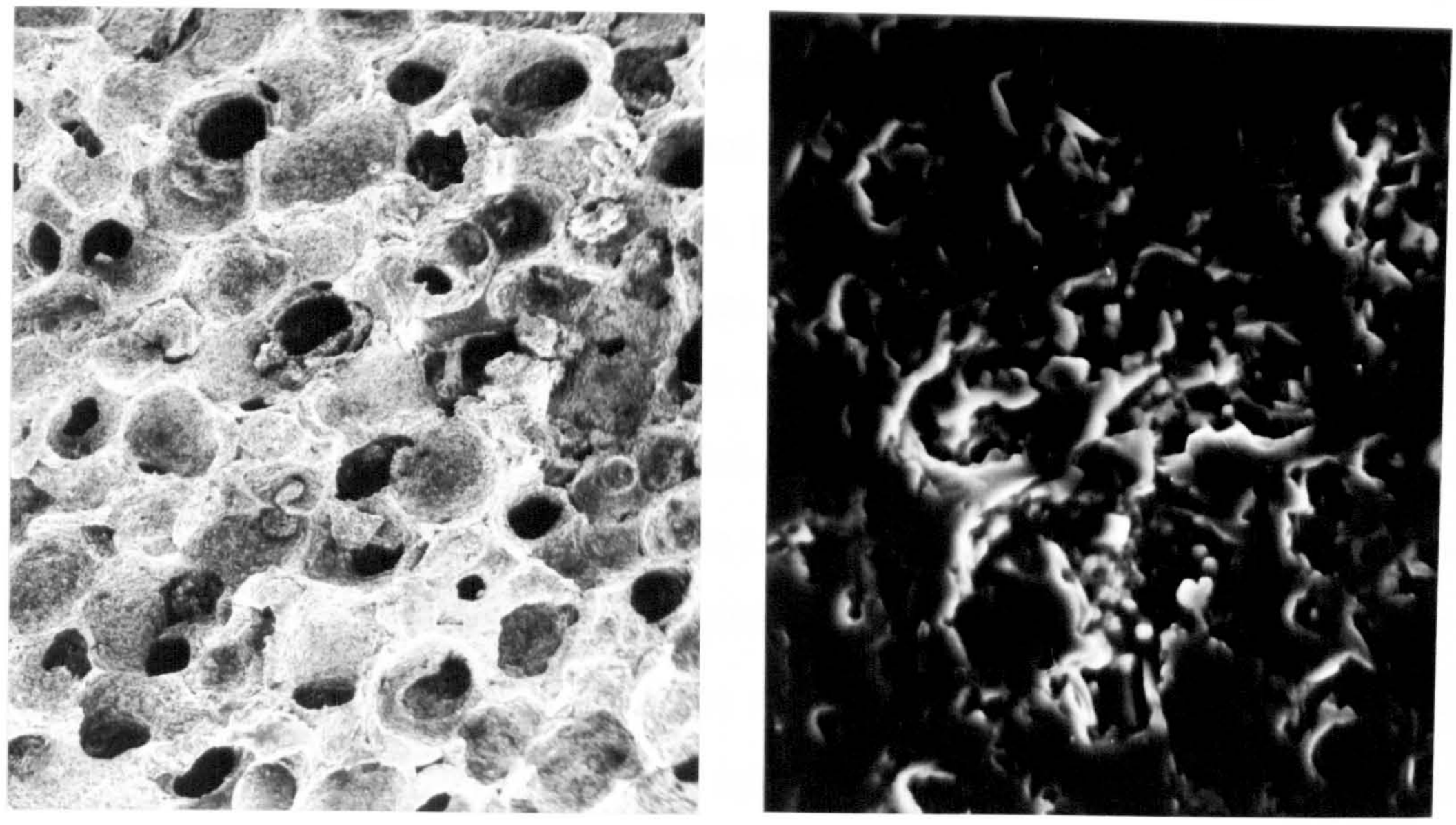


Photograph 2.1. Optical micrographs of polished surfaces of sintered dense HA (A) and HA-Spinel (B) materials. Interconnected pores were not found in the materials. Imaging area: 120 x 120μm (A) and 70 x 70μm (B). Magnification: x800 (A),: and x1250 (B)

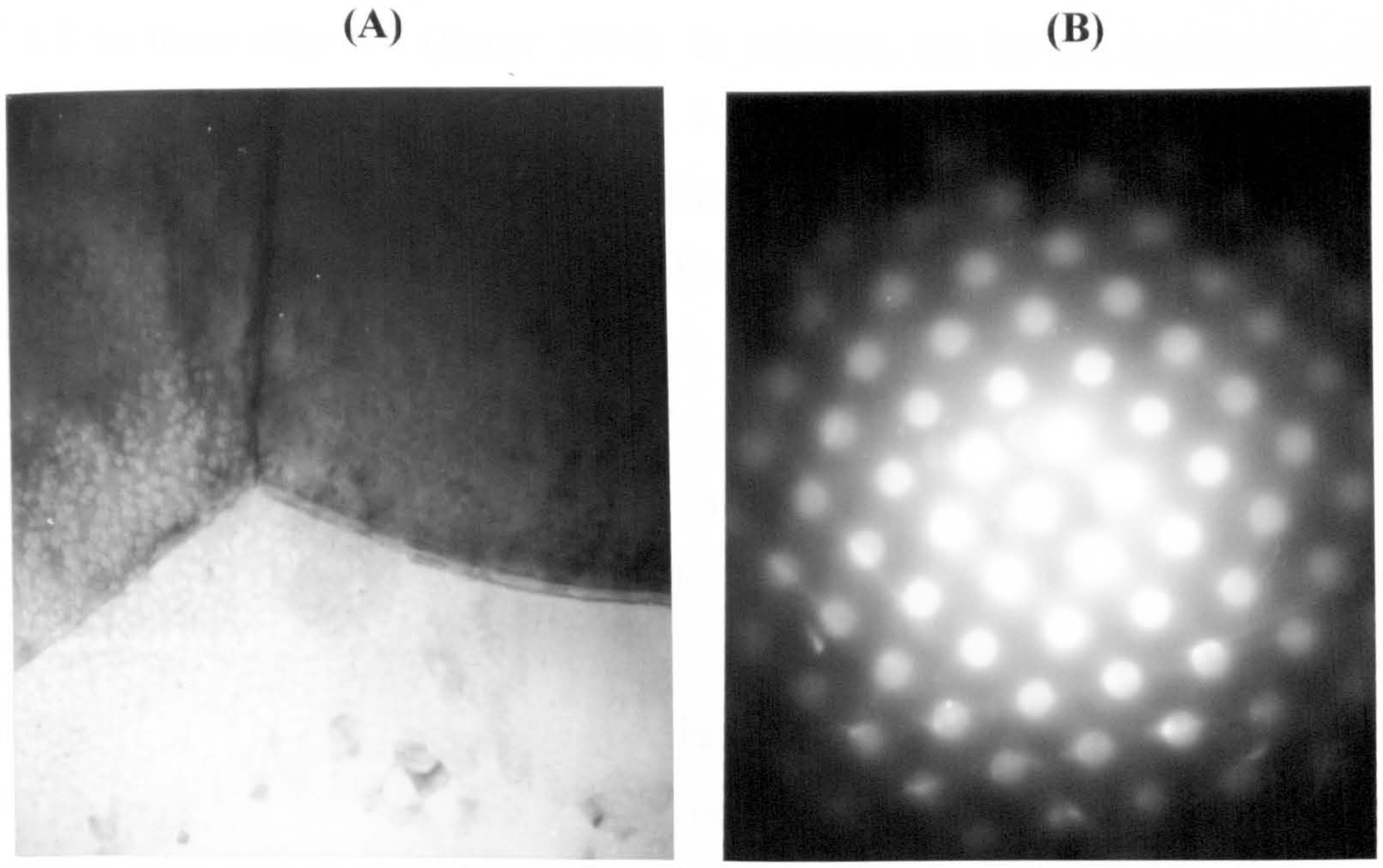
(A) (B)



Photograph 2.2. SEM photographs of HA powder (A) and Spinel powder (B). HA powder has a rather complicated morphology and the powders are assembled by smaller particles. Imaging area: 20 X 20μm (A and B). Magnification x3000



Photograph 2.3. SEM photographs of polished surfaces of dense HA-Spinel material (A) and porous HA material (B) (porosity: 45%). Porous structure was achieved in all porous materials. Imaging area: 20x20 μm (A) and 3.5x3.5mm (B). Magnification x3000 (A) and x60 (B)



Photograph 2.4. Transmission electron micrograph of argon ion thinned HA-Spinel material after sintering at 1450°C for 4hr. Two HA particles(crystalline) upper and one Spinel at bottom, x25,000 (A), and corresponding to the 111 lattice plane of HA crystal (B).

2.4 Discussion

In the process of preparation of calcium phosphate materials, the most important factors are particle size of the powders and the sintering temperature. During sintering, over-sintering may result in shape change, producing big crystals which result in brittle mechanical properties, and phase changes. It is difficult to obtain pure HA material. Usually TCP shows present in the sintered materials because of the phase transformation during sintering at elevated temperature. In my previous work, we found that most of the highly porous TCP material with pore size ranging from 100-600 μm could be resorbed within two years in man(Ruan et al, 1993b). In some cases, the speed of new bone forming was slower than the speed of the degradation of porous TCP implant. Thus a hole was left in the bone and a second operation to fill this in with the material was required.

HA is the only thermodynamically stable phase in calcium phosphate system in aqueous solution under normal conditions at pH of 6.3 (Bauer, 1990) . However, the exact stoichiometry of the HA in body tissue may vary in a very large range of Ca/P ratio of 1.3 to more than 4.0 (Bauer 1990). In addition, the normal ions in the chemical formula may be substituted by other ions. For example, Ca^{2+} may be partially substituted by K^{+} or Mg^{2+}. PO_4^{3-} may be replaced partially by SO_4^{2-} and CO_3^{2-} ions, and OH^{-} may be substituted by F^{-}. As described above, HA belongs to the hexagonal-rhombic prisms group with cell dimensions in the ranges of a=9.3 to 1.05nm, c=0.684-0.75nm and c/a=0.74-0.71, density 3.10g/cm^3. TCP belongs to the whitlokite structure group with cell dimensions of a=1.034nm, c=3.69nm, and c/a=3.57 (Heimke, 1984). Since sintering is normally done above 1150°C, cooling to room temperature results in phase transition from HA to TCP and a consequent 7.3% decrease in volume, therefore suitable techniques must be employed to counter that phenomenon if a stable material is required. Otherwise, the microstructure of the ceramic will be subject to over stressing and cracking. Also as indicated the CaO-P_2O_5 phase diagram in Figure **2.8**(de Groot et al, 1990), HA is a longitude line in the phase diagram. The transform temperature is around 1475°C. In practice, in all the manufacturing processes, it is difficult to control the influences of the manufacturing steps on crystalline transformation, hence from 1250°C the HA may decompose according to the reaction:

$$Ca_{10}(PO_4)_62(OH) \Rightarrow 2\beta\text{-}Ca_3(PO_4)_2 + Ca_4(PO_4)_2O + H_2O \quad \text{or}$$

$$Ca_{10}(PO_4)_62(OH) \Rightarrow 3\beta\text{-}Ca_3(PO_4)_2 + CaO + H_2O$$

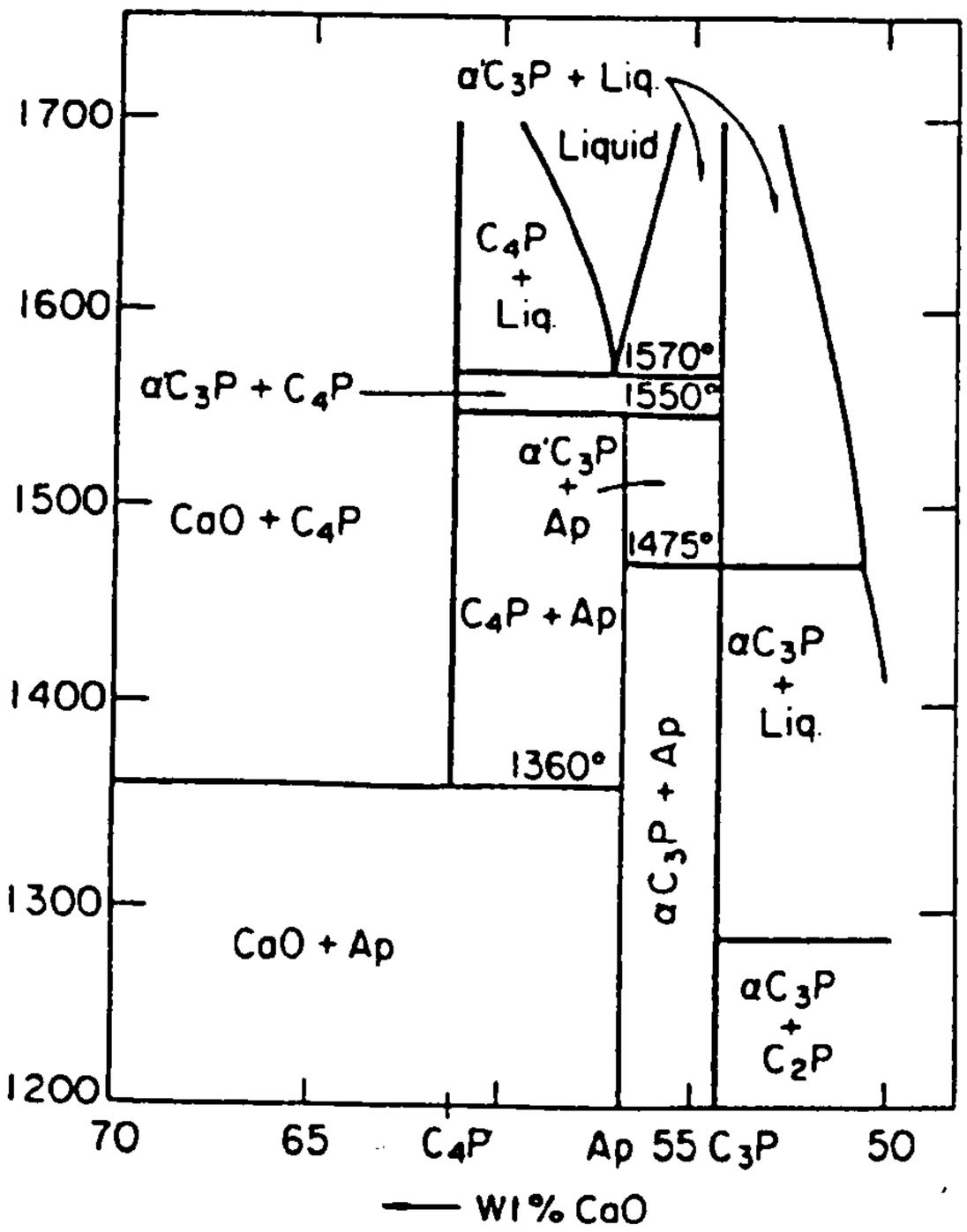


Figure 2.8. Phase diagram of the system $CaO\text{-}P_2O_5$ at high temperature (vertical axis: temperature °C, Ap=Apatite=Hydroxyapatite). (from de Groot et al, 1990)

The X-ray analysis of sintered HA and HA-Spinel shows that TCP is present in both the sintered HA material and to a great extent in the sintered HA-Spinel material. The former is a mixture of HA and TCP and latter is a mixture of HA, TCP, and Spinel and by TEM, we found that the lattice structure of the boundary between Spinel crystal and HA crystal is lack of regulation in terms of non-integrated lattice. TCP is unstable in the presence of water and reacts in time to produce HA:

$$2H_2O + 4Ca_3(PO_4)_2 \Rightarrow Ca_{10}(PO_4)_6(OH)_2 + 2Ca^{2+} + 2HPO_4^{2}$$

here the $Ca_3(PO4)_2$ is looked upon as a defective apatite (HA)(Ravaglioli and Krajewski, 1988). Since bone tissue and teeth are made up of an inorganic phase, based on calcium phosphate and in particular on HA, it is understandable that already by the beginning of this century, calcium phosphate was considered as the most suitable material to repair or even replace bone. With the development of better sintering techniques it became possible to produce calcium phosphate biomaterials for this purpose.

In practice, elimination of all pores in a sintered sample is rare, with the exception of liquid state sintering. HA-Spinel was sintered at 1450°C or above which is close to the melting temperature of calcium phosphate compounds according to the P_2O_5-CaO phase diagram. If liquid phase is present in the sintering material, a higher density close to the theoretical density may be obtained, i.e. liquid sintering, but phase transformation will be unavoidable. An aspect that must be noted is that although HA, TCP and Spinel are themselves non-toxic, the toxicity of the intermediate products must be tested. In the present research and in my previous work, X-ray diffraction did not show the presence of intermediate products between HA and Spinel or between TCP and Spinel (Wen et al, 1993; Ruan et al, 1993). Mechanical properties are affected by many factors, such as composition, crystal size, porosity, pore size as well as pore distribution, and processing parameters such as sintering temperature and sintering time. If other conditions are identical, the starting particle size and sintering temperature are critical. Many physical features and the phase structure and porosity of sintered materials are directly or indirectly associated with the sintering temperature. For solid state sintering, in general, porosity is decreased with increasing sintering temperature. Principally, sintering is always associated with a considerable volume shrinkage and density increase of the body concerned, because in compressing HA and HA-Spinel in a steel die, the compact densities are unlikely to reach more than 60% of the theoretical density. Thus during sintering at high temperature, both HA and HA-Spinel can decrease in volume by up to 50%, resulting in a linear shrinkage of about 15-21%. McGee et al (1974) demonstrated that by adding $MgAl_2O_4$ to HA and TCP materials, the mechanical properties of HA or TCP were significantly improved. I also demonstrated this during my previous work with HA materials (Ruan et al, 1993a). $MgAl_2O_4$ has a cubic structure with lattice constant of 0.8nm, and a density of $3.6g/cm^3$. Oxygen atoms construct the face centred cubic. Al atoms are in the octahedral sites and Mg atoms are in the tetrahedral sites. Figure **2.9**

gives the reproduction of the Spinel phase diagram in which it can be considered as a very stable interoxide compound. Table **2.5** summarises the main features and properties of the oxide (Heimke, 1984).

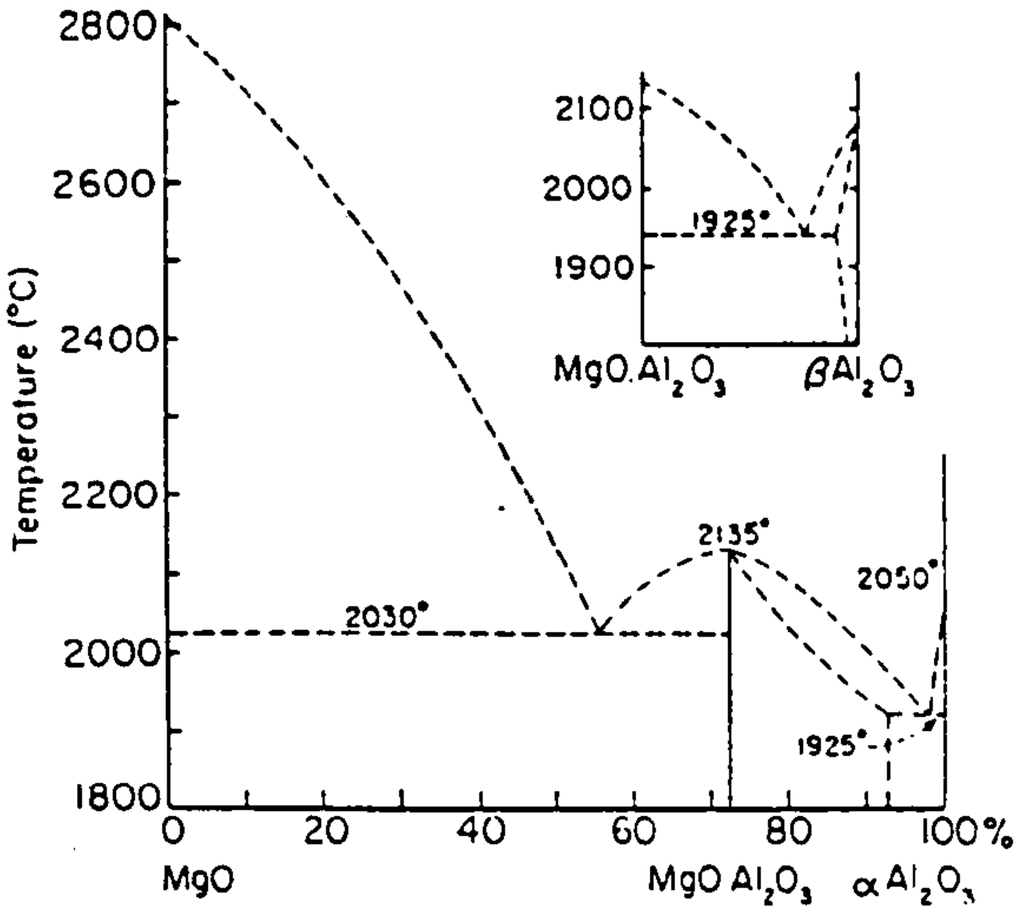


Figure 2.9. The Al_2O_3-MgO phase diagram. (from Heimke, 1984)

Table 2.5 Basic properties of $MgAl_2O_4$. (from Heimke, 1984)

Density (g/cm^3)	**Young's modulus** (MPa)	**Compressive strength** (MPa)	**Bending strength** (MPa)	**Hardness** (MPa)	**Thermal expansion** ($10^{-6}/K$)
3.60	$2.3x10^5$	1000	150	2070	6.7

The volume change of sintering materials originating from the transformation of the HA phase to TCP phase leads to stress in the sintered materials, therefore lowering the mechanical properties. On the other hand, by adding Spinel to HA the transformation of HA to TCP may be inhibited, the net structure of Spinel in the material could enhance HA and inhibit the propagation of cracks. Hence the mechanical properties would be improved by adding Spinel to HA(Wen et al, 1993). If the ceramic is made flawfree, then it becomes very strong both in tension and in compression. However, it is very difficult to eliminate pores in the sintered materials and the mechanical properties are extremely affected by pore morphology and pore distribution. The relation between compressive strength (s_c) and the portion of the total volume occupied by pores, V, may be described with the following equation (Thümmler and Oberacker, 1993; de Groot et al, 1990):

$$\mathbf{s_c = 700e^{-5V}} \qquad (V<50\%)$$

A similar relation holds for tensile strength, $\mathbf{s_t}$, and microporosity, $\mathbf{V}$.

$$\mathbf{s_t = 220e^{-20V}} \qquad (V<5\%)$$

It is obvious that small variations in microporosity have a much larger influence on the tensile strength than on the compressive strength. Mechanically, HA and TCP can only withstand compressive forces and are easily damaged by tensile or great bending forces. In my research, the porous HA materials, however, are very much weakened, from a mechanical point of view, by the presence of pores. Therefore the porous HA material is only suitable for filling bone vacancies without load bearing. Compared with metal materials, the much lower heat conductivity of the HA or TCP ceramic materials, result in the requirement of long cooling time from sintering temperature to room temperature. In practice we often encountered this problem and to avoid the presence of microcracks in the materials, the cooling time was increased to more than 70hr (cooling rate: ~20°C/hr). In the present research, the compressive strength and bending strength of dense HA-Spinel material (see table **2.4**) was greater than or equal to that of human bone(compressive strength: 130-160 MPa). However its toughness of 1.6~1.9MPa $m^{1/2}$ (Raun et al, 1993b) is only 1/4~1/6 of the toughness of human bone (2~12 MPa $m^{1/2}$) (Ravaglioli and Krajewski 1990), so its application is considerably limited.

Comparing the data on bending strength in table **2.3** with that on compressive strength in table **2.4** shows that the bending strength had been improved by adding the Spinel component, whereas there was only a small increase in compressive strength. This difference is due to the different stress conditions produced by the tow tests. Within the bending specimens there are tensile stresses, which do not occur in the compression test specimens. The presence of tensile stresses leads to crack propagation, which is the primary cause of failure in such tests. Whether a crack propagates depends on the local tensile stress, the length of the crack and the mechanical properties of the materials. For a given crack length, the tensile stress required to produce propagation is proportional to the square root of the elastic modulus; thus cracks which propagate through low modulus material may by arrested in a high modulus material. The elastic modulus of Spinel (230 GPa) is considerably higher than that of HA (40-117GPa) and the presence of an increasing amount of Spinel will inhibit crack propagation in the bending tests and

therefore increase the bending strength; this mechanism is not applicable of compression where there are not tensile stresses and the mechanism of failure is quite different. The effectiveness of the crack inhibition by Spinel also depends on the structure of the materials. HA particles are surrounded by Spinel particles, which are constructed of a net structure and this structural combination will make the crack inhibition by Spinel particularly effective.

Normally in the porous materials, if the porosity is over 9%, the pores in the porous material are connected with each other(Williams 1960; Huang 1983; Thümmler and Oberacker, 1993;). Average pore size and pore size distribution can maesured by microscopy. These variables can also be measured by mercury intrusion porosimetry (Sibilia, 1996). Mercury has a high contact angle and it will not enter pores without added pressure. The limitations of mercury intrusion porosimetry include: (a), pore size is limited in the range of 3-300μm; (b), all pores are presumed to be as cylindrical in shape. Pore size also can be determined by gas adsorption method (Everettand and Stone, 1958; McEnaney et al, 1997). Basically, the amount of gas needed to form a monomolecular layer on the solid surface can be determined from the volume of gas absorbed to the surface, and surface area is calculated by the absorbed mono-layer molecule volume over by the section area of a single molecule. The pore diameter may be calculated mathematically by the relationship between surface area and diameter. Gas adsorption is especially useful to measure small pores (with pore size smaller than 50μm).

Various approaches demonstrate that bone osteo-conductivity depends on the porosity, both on pore size and on pore communication (Linnad and Chao, 1986; Beirne and Greenspan, 1985; Donath, 1990), which is a prerequisite for tissue ingrowth. From long term observations, both in animal experiments and in clinical cases, researchers get the impression that bone formation ceases at a certain distance from the surface, whereas vessels and fibroblasts still continue to invade the deeper layers (Kenney et al, 1985;). This indicates that the inductive stimuli come from the surrounding bone and at a depth of several millimetres into the material.

The in vivo behaviour of calcium phosphate implants depends on a variety of factors, among which are the Ca/P ratio, the crystallographic structure, and the degree of porosity (Kushitani et al, 1994; Ohgushi et al, 1994; Yoshikawa et al, 1994;). The specific physiological conditions, like biomechanical or structural requirements, may also

decisively influence the biological response. It nevertheless appears that maximum osteogenic activity and strength of the ceramic-bone link is not achieved by pure HA, but by a mixture of HA containing 7% β-TCP(Krajewski et al, 1988). In the case of porous ceramics, when an object composed of particular calcium phosphate, such as HA, is surrounded by connective tissue, a reticulated structure develops accompanied by some degree of crumbling of the ceramic in the area surrounding the periphery, unless mineral osteointegration takes place. Krajewski et al, (1988) found that the same microporous material can absorb Ca^{2+} ions from the physiological environment down to a depth of some 400 μm.

Ravaglioli and Krajewski (1992) suggested that porous ceramic with pore dimensions of 200 μm is enough to allow the growth of osteons and vascular connective tissue, and provide a lattice and cellular environment able to give rise to the formation of appositional bone by a process that resembles the natural process. In contrast cancellous bone is converted to compact bone through progressive deposition of lamellae on to new bone - a process which develops a series of osteons. The growth of bone taking place in ceramic implants seems to be conditioned by the type of porosity as well as by the pore dimensions. The search for a relationship between these two factors was the aim of a study by Ishang et al (1997). However, the mechanisms regulating the effect of porosity or pore dimension on the depth of ingrowth of bone tissue are not yet clear. An appropriately porous ceramic will create skeletal and environmental conditions able to favour such deposition. In order to promote the development of osteons, the pores must have dimensions over 100μm because the mean dimension of Haversian system is about 100μm (Ravaglioli and Krajewski 1992; Van Raemdonck, 1984). For an estimation of the optimal pore dimensions and the most suitable porosity percentage, we must take account of the effect on the growth of bone and also on blood flow which provides the calcium and phosphate ions that are needed both for the mineralisation of the organic matrix and for the supplement of nutrition. Klawitter (1970) measured the depth of the mineralised bone grown into pellets made of porous calcium aluminate implanted into canine femora and left in position for up to 22 weeks. Figure **2.10** shows the variation of the rate of bone growth he measured as a function of the pore dimension. From this graph it is possible to infer that pores with diameter significantly less than 100 μm do not

permit cell and tissue colonisation. By animal tests (mongrel dogs), Kuniomi and Yoshio (1990) stated that HA with 300 to 600μm pores provided the best circumstances for achieving osteogenesis. The intercommunication of these pores enables bone to grow in, therefore, in a limited distance. It is evident that increased pore dimensions favour the ingrowth of bone. However, the pore dimensions cannot be increased to the extent that they weaken the strength of the implant material, consequently making it unsuitable for practical application. It has been generally recognised that long winding pores cannot be completely filled with new forming bone because nutrition and oxygen supply are inadequate at the far end of the pores. Based on the above literature, and consideration that the growth rate of new forming bone should match the degradation rate of the porous material, and on the suggestion of clinical surgeons as well, the porous materials in this research have pore structure and pore sizes which mostly ranged from 100 to 500μm, with the average dimension of pores being 315μm.

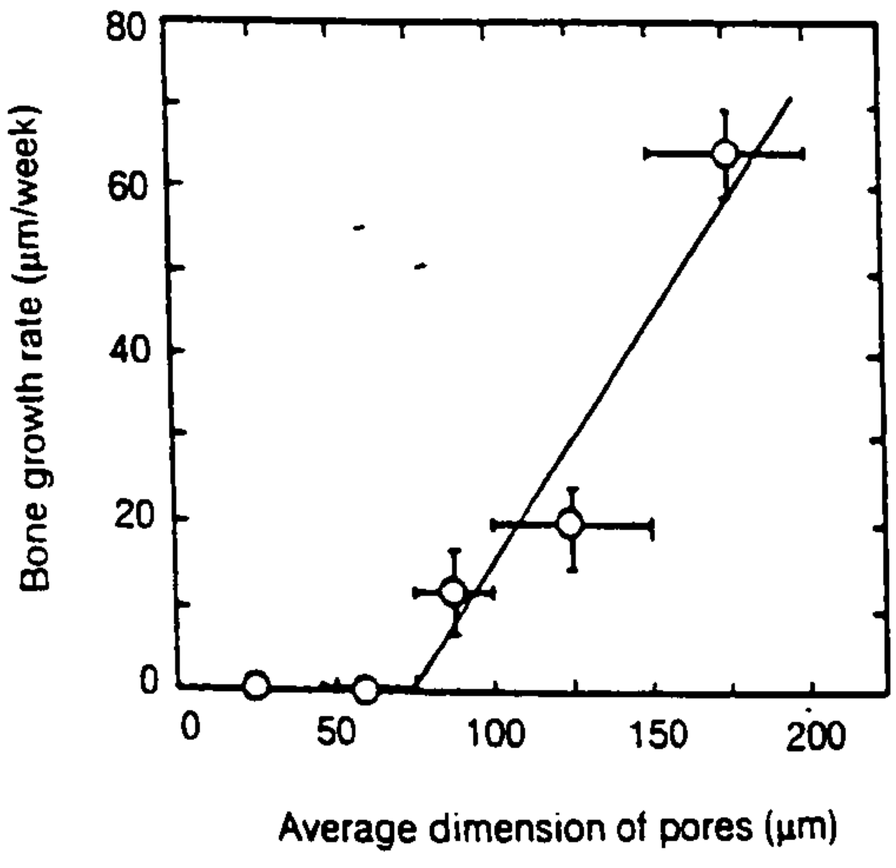


Figure 2.10. Variation of bone growing speed as a function of the pore dimension under constant porosity condition. (from Klawitter, 1970).

Chapter 3 Cell Distribution And Growth Through HA Based Biomaterials

3.1 Introduction

The ability to observe the behaviour of a specific cell type in a well controlled environment, is a major attraction for using cell culture to investigate compatibility of biomaterials. Interaction at both the cellular and molecular levels can be examined in response to a material or its component parts. The cell reaction to implanted materials usually results in a series of complex, integrated responses which occur over a period of months. An important consideration is that the cells used should represent those found at the potential implantation site, for example, osteoblasts provide an ideal model for orthopaedic materials. Another attraction in the use of cell culture lies in the ability of cells to propagate and survive long term storage ensuring consistent experimental reproducibility.

Many studies have been performed on calcium phosphate based biomaterials, mainly HA and β-TCP and α-TCP, in vivo, since 1982 (Klein 1985; Daculsi et al, 1990; Kotani et al, 1991; Bagambisa and Joos, 1990; Jarcho et al, 1977; van Blitterswijk 1985), and in vitro, during past few years (Kokubo et al, 1987; Seitz et al, 1982; Gomi et al, 1993), in order to investigate their biological characteristics, biocompatibility and potential clinical use. Successful application of grafts based on calcium phosphate materials has been achieved by several groups in the clinic (Bucholz et al, 1987; Caulier et al, 1995; Denissen et al, 1984). My previous work has investigated the biodegradation behaviour as well the bone bonding behaviour of HA based materials during in vivo implantation in New Zealand Rabbits and clinical application (Ruan et al, 1993b; Weng et al, 1993).It usually took 6 months for the dense biomaterials to integrate with natural bone, and the living tissue can grow deeply in the porous materials in 16 weeks. Despite much research, the processes involved in the formation of new human bone after implantation of HA materials are still not clear, and inflammation during the early period of implantation often takes place(van Blitterswijk et al, 1990). Histochemical observations in animal tests have suggested that the degradation of HA seems to be necessary for osteoconductivity and remoulding of the fibrous connective tissues to form natural bonding with implants (Edwards et al 1997; Thomas et al, 1987; Hong et al,

1992). Experiments in vivo do not allow detailed examination of the responses of special cells or tissues to the biomaterials, in order to determine the events and factors which influence the interactive process. For this reason model cell culture systems in vitro provide the majority of the information available at present. There are usually two types of cells, osteoclasts and osteoblasts, involved in evaluation of the compatibility of the potential implant materials in vitro. Osteoclasts often display degradative behaviour towards implant materials and researchers have demonstrated that HA, TCP and other bioactive glasses were phagocytosed by osteoclasts (de Bruijn et al, 1994; Ross et al, 1996). The reaction of macrophages, monocytes as well as other lymphocytes to the implant material is the foreign body reaction, and as a consequence, acute inflammation may be produced in the tissue surrounding the implant. It is difficult to determinate quantitatively the rate of degradation of calcium phosphate biomaterials in vivo as degradation in human body depends on so many factors, such as chemical composition of the implant materials (Klein 1985; Ong et al, 1995; Jarcho, 1981), shape of implants (Nordsletten et al, 1996), microstructure of the materials, i.e. porosity (van Blitterswijk et al, 1985; Klein et al, 1983), pore size, pore shape(Cheung et al, 1989), and even the roughness of the material surface (Radin and Ducheyne, 1994; Gregoire et al, 1990). All the biological reactions between the implant and the tissues take place at the material surface, which is composed of the outside surface and the walls of the pores, therefore, the surface condition is of crucial importance.

Compared with studies of the response of osteoclasts to calcium phosphate biomaterials , there has not been as much work dealing with the responses of osteoblasts to calcium phosphate ceramic biomaterials in vitro(Matsuda et al, 1987; Ziats et al, 1988), and, even less work on the reaction of human osteoblasts to highly porous calcium phosphate biomaterials (de Bruijn et al 1994), although a lot of dense and porous calcium phosphate materials have been tested in vivo. In principle, bone bonding between tissue and implants depends on the results and consequences of the responses of the osteoblasts to the implant materials. This includes the ability of the cells to synthesise extracellular matrix components for example collagen.

In this part of my research programme I have investigated the biocompatibility and interaction of HA based biomaterials with the bone forming osteoblast cells in vitro using two lines of immortalised osteoblasts, FFC and THO cells, isolated from rat

neonatal calvariae and human adult jaw tissue, respectively. These cells were transfected by calcium phosphate precipitation of SV40 DNA and have been used extensively by our group for biocompatibility studies of orthopaedic materials (Morrison et al, 1995; McKay et al, 1996; Macnair et al, 1996; Macnair et al, 1997). In the present research, dense HA biomaterial was modified by the addition of Spinel ($MgAl_2O_4$) which improves the strength and wear resistance of the resulting dense material (Ruan et al, 1995). Thomas (1974) confirmed that $MgAl_2O_4$ was a nontoxic, bioinert material, although to some degree, the bioactivity of HA was inhibited by combination with $MgAl_2O_4$ (Mcgee, 1974; Blumenthal, 1989). However, the mechanism of inhibition is not clear and the research was mainly carried out in vivo.

The goal of this part of my research was to evaluate the proliferation and distribution of THO cells on the dense HA-Spinel biomaterial and on highly porous HA materials that have been successfully used in the clinic for over the past nine years, and tested in vivo (Ruan et al, 1993; Wen et al, 1993). Cell proliferation on the materials was assessed by measuring total protein content by the method of Lowry and coworkers (1951). Previous workers (Doostdar et al, 1990; Hanthamrongwit et al, 1996) have shown that measurement of the total cell protein in proliferating cell cultures correlates well with cell numbers throughout the log phase of growth. It is recognised, however, that this measurement will also take into account increases in intracellular protein content due to the cells growing in size without division, but this is considered to be a minor contribution under conditions of log growth. As the osteoblasts approach confluence they produce extracellular matrix on the materials. This is composed predominantly of type I collagen and although it will contribute to the overall protein content of the cultures it will not be detected by the Lowry assay. This method detects tyrosine, tryptophan, and phenylalanine amino acid residues on protein molecules and as collagen is mainly composed of glycine, proline and hydroxyproline, it is not detected.

The protocol used for the Lowry assay ensures that only attached cells are measured, and it is assumed that to remain attached to the materials the cells must be viable. Thus, the protein content is equivalent to number of viable cells. This is a major advantage over the use of cell numbers directly. To use this approach materials would have to be treated with trypsin to release the attached cells and it is likely that a proportion of cells would be damaged or killed by the trypsin, and also it would be

difficult to ensure that cells embedded deeply within the materials were released by the enzyme. For these reasons the measurement of the total protein content is thought to be a more accurate and convenient measure of cell proliferation.

Cell morphology on the dense material and in the deep pores was investigated by confocal laser scanning microscopy (CLSM).

3.2 Materials and methods

3.2.1 Preparation of material Samples

Both porous HA and dense HA-Spinel were prepared by the chemical synthesis method previously described (Ruan et al, 1992) and the final amount of Spinel in the sintered material was 25 wt%. The process for synthesis of the materials is schematically outlined on Figure **2.2**. For porous HA the sintering temperature was 1250°C and it has a relative density of 40-50%. The HA-Spinel was compacted as described, followed by sintering at 1450°C for 2 hours. The relative density of the sintered material is over 93%. After sintering, both materials were polished with sand paper. Type 600# was used for the porous material, and type 1200# for the HA-Spinel

Before cells were cultured on the materials, the material specimens (diameter 12mm and thickness 3mm) were immersed in boiling water for 1 hour, and sterilised by autoclaving (121°C, 1.2kg/cm^2 pressure, 30min), then immersed in complete culture media for 48 hours. Subsequently, the immersed materials were washed with sterilised distilled water and autoclaved again, thereafter being stored at room temperature until they were to be used for cell culture.

3.2.2 Culture of human and rat osteoblast cells

Immortalised human osteoblast cells(THO) and immortalised rat osteoblast cells(FFC) have been well established in our laboratory. They provide several useful properties when used in conjunction with biocompatibility tests (Macnair 1996). They are easily preserved in liquid nitrogen and can be readily resuscitated from the frozen condition. The recovery process of the cells is simply described as follows: 1ml quantities of suspended cells frozen at a concentration of 5x10^6 cells/ml were retrieved from the liquid nitrogen storage. The cell suspension was thawed out rapidly at 37°C before being added to 5ml of complete medium in a 25 cm^2 flask. The cell cultures were placed in a 37°C incubator in an atmosphere of 5% CO_2 in air until confluent. FFC cells were grown in Dulbecco's modification of Eagle's Minimum Essential Medium(DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), penicillin (50 IU/ml), streptomycin (50 μg/ml). THO cells were grown in Ham's F-10 medium supplemented with 10%(v/v)

FCS, ascorbic acid(50mg/l) and the antibiotics as described above. To passage the cells, a 1:5 dilution of 0.25% (w/v) trypsin in 0.02% (w/v) versene in phosphate buffered saline (PBS) was prepared. The culture medium from the 25 cm^2 flask was discarded and the cells were washed twice with the 0.02% versene in order to remove the FCS as this would inhibit the action of the trypsin. 1ml of the trypsin/versene solution was added and left in contact with the cell layer in the incubator until the cells had detached from the surface. This required about 1-2 min of exposure. Once the cells had detached , the action of trypsin was inhibited by adding 9ml of DMEM containing 10% (v/v) FCS for FFC or Ham's F-10 containing 10% (v/v) FCS for THO. To ensure that the cells were evenly suspended, the contents of the flask were drawn up and down using a 5ml pipette. The final step in the routine was that the cell suspension was split at 1:10 for FFC or 1:3 for THO respectively, and re-seeded into 25 cm^2 flasks. For FFC cells, the procedure was performed in a class I flow cabinet, and for THO cells, in a class II flow cabinet.

3.2.3 Growth curves of human and rat osteoblasts

In order to establish the relationship between cell proliferation and culture time, THO at passage 8 and FFC at passage 30 were seeded in the 25 cm^2 flasks at the seeding density of 10^6 cells per flask(~$4x10^4$ cells/cm^2) for THO and $5x10^5$ as well as 10^6cells per flask($2x10^4$ cells/cm^2 as well as $4x10^4$ cell/cm^2) for FFC. Culture conditions were as described. The culture media were renewed every second day and at 24hr intervals four flasks in each cell group were taken out off the incubator. The culture medium was discarded and the cells washed with PBS buffer three times. 1ml 0.5 M NaOH was added to each flask to digest the cells for 18 hr (over night), and then the samples were stored at -18°C until analysed for protein content by the Lowry assay (Lowry et al. 1951).

3.2.4 Cell culture on calcium phosphate biomaterials

Before cells were cultured on the materials, samples (12mm diameter and 3mm thickness) of the porous material and the HA-Spinel dense samples were attached to the bottom of 35mm Petri dishes using medical grade adhesive tape, preventing the discs moving in the culture medium. THO cells (passage 12) or FFC cells (passage 30) were added to the surface of the materials at a seeding densities of $5x10^4$ per cm^2 (THO) and $2.5x10^4$ per cm^2 (FFC) in a minimal volume of medium (approximately 0.5ml). One hour

later, when the cells had attached to the surfaces, the specimens were fully immersed in the appropriate culture medium. Samples were incubated at 37°C in a atmosphere of 5% CO_2 in air. Cells on materials were cultured for 4 days then washed with PBS. The cells were fixed on the samples with 10% phosphate buffered formaldehyde(PBF) for 48 hours, then washed thoroughly with distilled water and prepared for CLSM examination of the morphology, distribution and growth of the cells on the materials.

3.2.5 Total protein determination of cells cultured on materials

To investigate cell proliferation and growth on the materials, both THO cells and FFC cells were cultured on the dense HA-Spinel material discs placed in 24 well plates. For THO the seeding density was 10^5 per well ($5x10^4/cm^2$) and for FFC $5x10^4$ per well ($2.5x10^4/cm^2$) and 1hr after seeding, 1.5 ml culture medium was added to the specimens and the samples were incubated as before. At 24 hr intervals the medium in the each row(4wells) was removed and the cells washed with PBS thoroughly to remove all traces of the medium. Then this row of wells was left dry and the plate returned to the incubator. On the eighth day, the final row was taken, and the cell protein in all the wells was solubilised using 1ml 0.5 M NaOH and total protein content determined by the Lowry assay. For comparison, a control group of cells was cultured in the 24 well plate (made of polystyrene) without materials.

3.2.6 CLSM investigation and SEM

Before examining cells cultured on the materials, a material disc was stained with 0.1% w/v ethidium bromide to test the possibility that the material would be stained by the dye. CLSM observation showed that there was no reaction between material disc and staining reagent. Cells were visualised in the CLSM studies by staining the samples with 0.1% w/v ethidium bromide for 6 min. Samples were examined using a Leica CLSM using a x25/0.75 water immersion lens. For cell distribution studies, the 488nm excitation line from an argon ion laser and 580 nm emission were used. The beam splitter was set at 510 nm to reject light below 510 nm and allow light above 510 nm to pass through. The surface was located and images collected at 10 μm intervals into the materials for quantifying cell number. The structure of the surface of the materials was investigated in reflectance mode using 514 nm excitation and 530 nm emission, with the beam splitter set

at neutral density. THO cells on both dense HA-Spinel material and porous HA material were counted by CLSM observation after culture for 96 hours for the comparison of cell growth on different materials. During cell counting, three specimens of each material and five counting areas with size 200μm x 200μm on each specimen were randomly chosen. For SEM characterisation of the material surfaces, the dense and the porous samples were mounted on carbon adhesive discs on 12.5mm aluminium stubs and sputter coated with gold. Examination was carried out on a Jeol JSM 840A SEM.

3.2.7 Analytical measurement of cell protein by the Lowry Assay.

The procedure for total protein measurement by the Lowry assay is simply described as follows: On the day of the assay, two solutions were made up:

Solution A: 1ml 1%(w/v) $CuSO_4$
1ml 2%(w/v) Na-K tartrate
98 ml 2%(w/v) Na_2CO_3

Solution B: 4-fold dilution of Folin's reagent

Before testing the samples, the method has to be standardised using a series of solutions (0-200μg/ml in 0.5 NaOH) of bovine serum albumin (BSA). 500 μl of the digested cell solution(in 24 well flasks) were placed in test tubes from each flask. To each of the standards and samples, 5ml of solution A was added and mixed. After 10min at room temperature, 0.5ml of solution B was added and mixed thoroughly. Both standards and samples were left for a period of 30min and the absorbance measured against distilled water at 725nm using an UV2101 PC Scanning Spectrophotometer.

3.2.8 Statistics:

All measurements were collected and expressed as means +/- standard deviations. Single factor analysis of variation [ANOVA] was employed to assess the statistical significance of results for all the experimental data.

3.3 Results

3.3.1 Growth curves and cell morphology

Growth curves of THO and FFC are shown in Figure **3.1** and Figure **3.2**. It seems that during cell proliferation and growth, both THO and FFC cells have an obvious latent phase, logarithmic growth phase and confluent phase. The growth curve shape was related to seeding density. Higher seeding density resulted in shortening the latent phase because there were more cells entering the logarithmic phase at the same time. Both human osteoblasts and rat osteoblasts are anchorage-dependent cells. After cell seeding, as observed by optical microscopy, THO cells began to attach to the flask surface within 10min and FFC cells began to attach to the flask surface within 5min. Both THO cells and FFC cells were transparent and had long processes extending from the cell body, which had a diameter of 10-20μm. With the increase in cell density, cell size changed and cells were crowded together, (Photograph **3.1.)**. THO cells can only reach 75-85% confluence while FFC can reach 100% confluence. If cells were held at confluence without being passaged, they peeled off the flask surface and died.

3.3.2 CLSM observation and SEM

Photograph **3.2** shows the polished surface structure of the dense HA-Spinel and porous HA materials examined in reflectance mode by CLSM. The CLSM observation showed that pores were present in the both HA and HA-Spinel material and were roughly round in structure. As observed by CLSM, immortalised human and rat osteoblast cells grew well on the surfaces of both the porous and dense materials. Both cell types appeared to spread out and flatten on the platelike surface areas of the materials. Cell morphology studies showed elongated ellipses, with long processes extending from the cells. In the porous HA cells grew along the walls of the pores and penetrated down to the bottom of many pores. There were some changes in cell morphology depending on the shape of the pore wall, but essentially cell morphology on the bottom of the pores remained the same as that on the surface. Photograph **3.3-3.5** show the morphology of cells grown on the dense material and on/in the porous material.

3.3.3 Cell proliferation and growth on dense HA-Spinel and porous HA biomaterials

Table **3.1** shows the quantitative distribution of the human THO cells on both materials after 4 days in culture. The total cell number growing on each material is not different (153 on the dense material compared with 142 on the porous material). However, the distribution of the cells is very different. On the dense material cells penetrated only to a depth of 40μm from the surface, whereas on the porous material cells were detected in pores at depths of 120μm from the surface. A similar result was obtained with FFC cells after 4 days in culture on the dense and porous materials, and the total cell number growing on each materials was about 2.5 times over that of THO cells when the seeding density of both cells was the same. It was difficult to accurately quantify the FFC cells on the materials as there were too many to count. Growth curves of THO and FFC cells are shown in Figure **3.1** and **3.2** respectively. It seems that the shape of the growth curve of FFC cells was governed by seeding density. When the cells become confluent cell death increased, particularly with THO cells. When the growth of the two cell types was measured quantitatively in terms of protein content on the materials it was found that adsorption of protein from the serum supplemented medium onto the porous material occurred to such an extent that it interfered with quantification of cell growth. In contrast, there was little if any adsorption detectable on the dense material. From the results of the protein assay, both human and rat osteoblasts grew more rapidly between 0 and 96 h on the dense material than on polystyrene, but growth on the polystyrene superseded that on the HA-Spinel after 96h in culture (see Figure **3.3**). Similar results were obtained for immortalised rat osteoblasts. The difference in growth rate on the two surfaces was exaggerated with the faster proliferating FFC cells.

Table 3.1. Numbers of immortalised human osteoblasts (THO) growing on dense HA-Spinel and porous HA biomaterial by CLSM observation. Cultures were initiated on the material specimens at a seeding density of $2.5x10^4/cm^2$ and incubated for 98 hours. Data were collected from five dense material specimens and five porous materials, and three areas on each specimen were randomly selected. Counting imaging area: 200 x 200μm.

Materials	Cell Number +/- SD
On dense HA-Spinel	153+/-26
On Porous HA	142+/-33

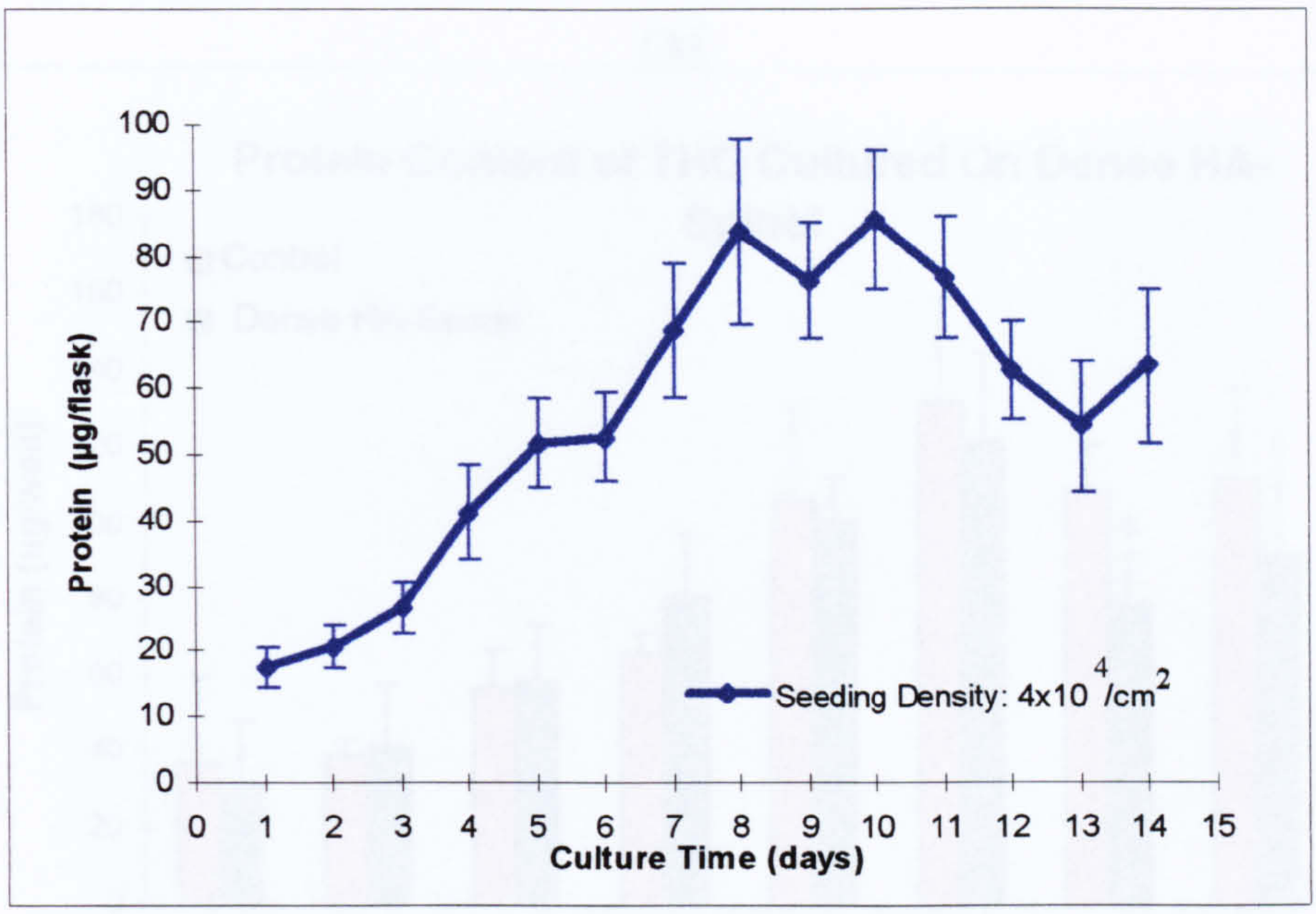


Figure 3.1 Growth curve of immortalised human osteoblast (THO) on polystyrene culture flasks. Culture were initiated in standard flasks (25cm^2 per flask) at a seeding density of 4x10^4/cm^2. Total proteins were measured by Lowry assay at different culture time. Results are mean +/- SD mean, n=4.

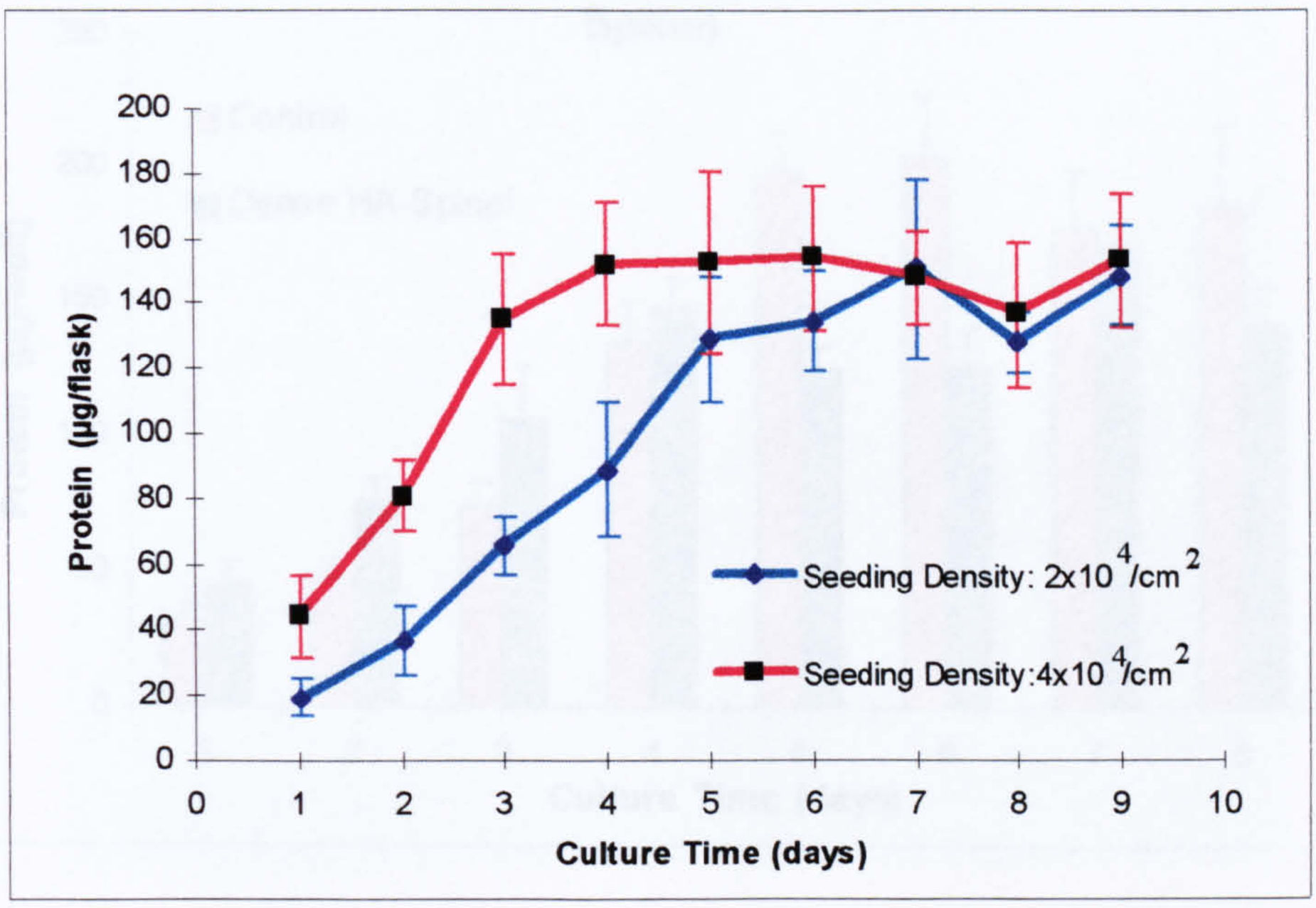
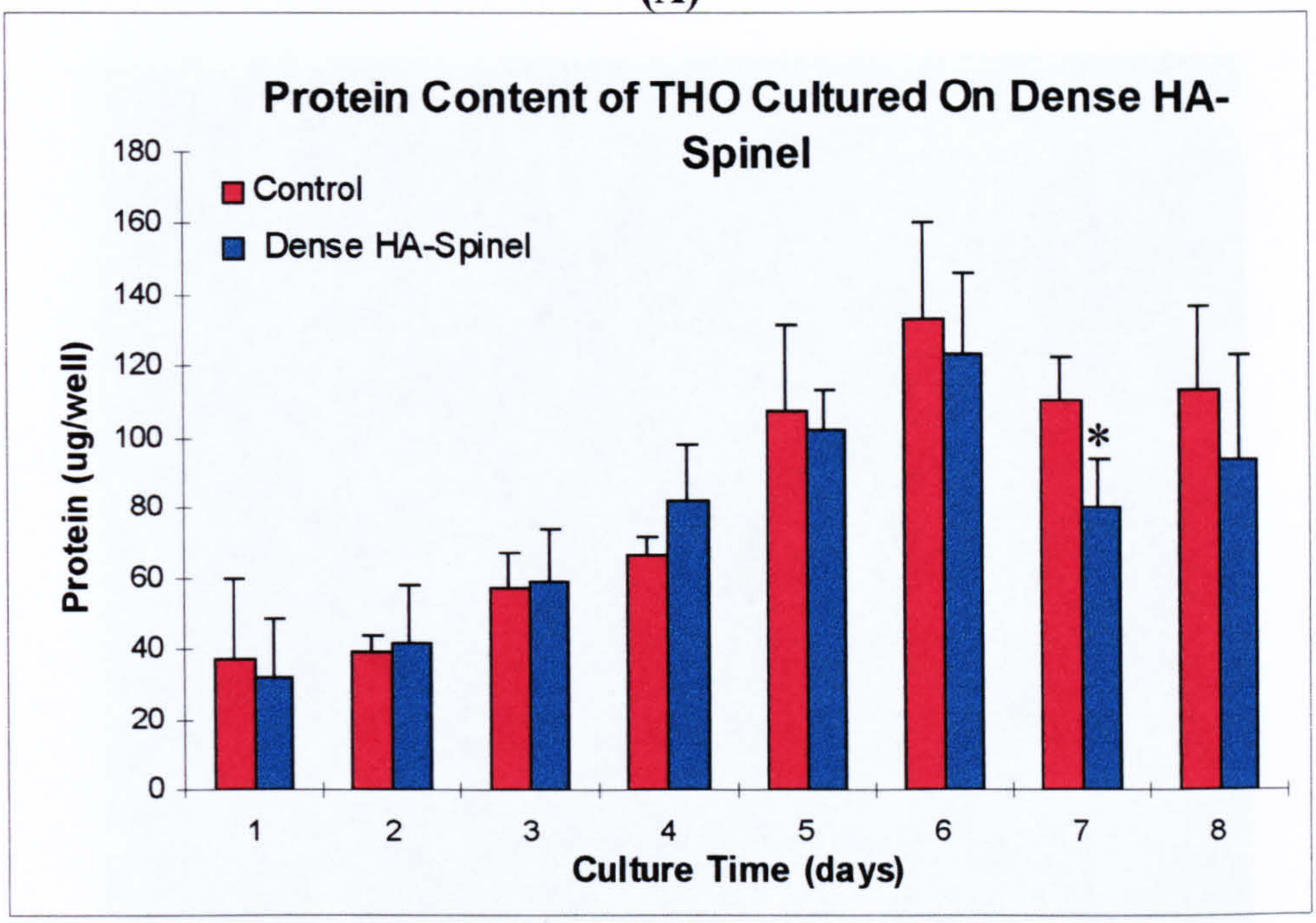


Figure 3.2 Growth curve of immortalised rat osteoblast (FFC) on polystyrene culture flasks. Culture were initiated in standard flasks (2.5cm^2 per flask) at a seeding densities of 2x10^4/cm^2 and 4x10^4/cm^2. Total proteins were measured by Lowry assay at different culture time. Results are mean +/- SD mean, n=4.

(A)



(B)

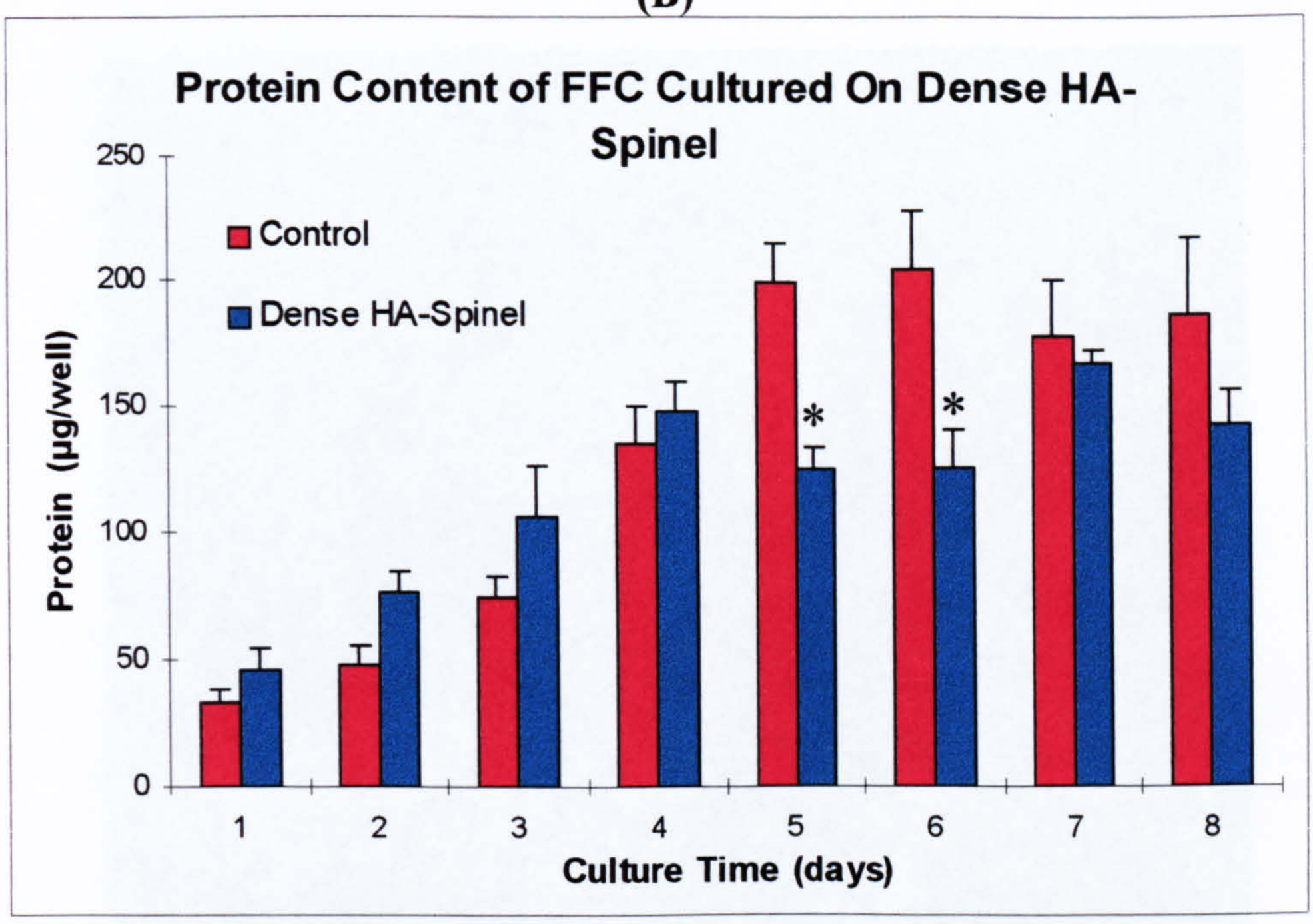
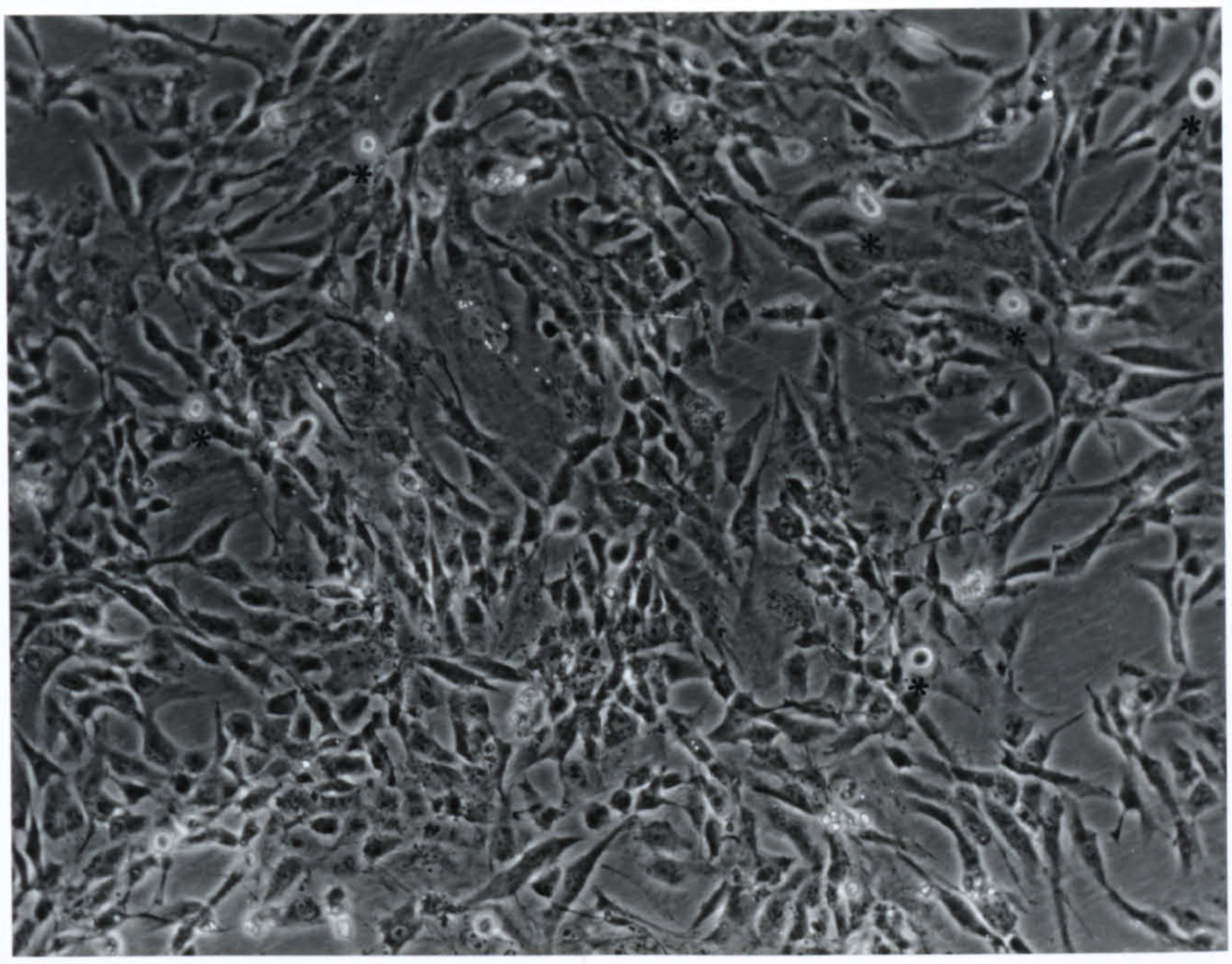
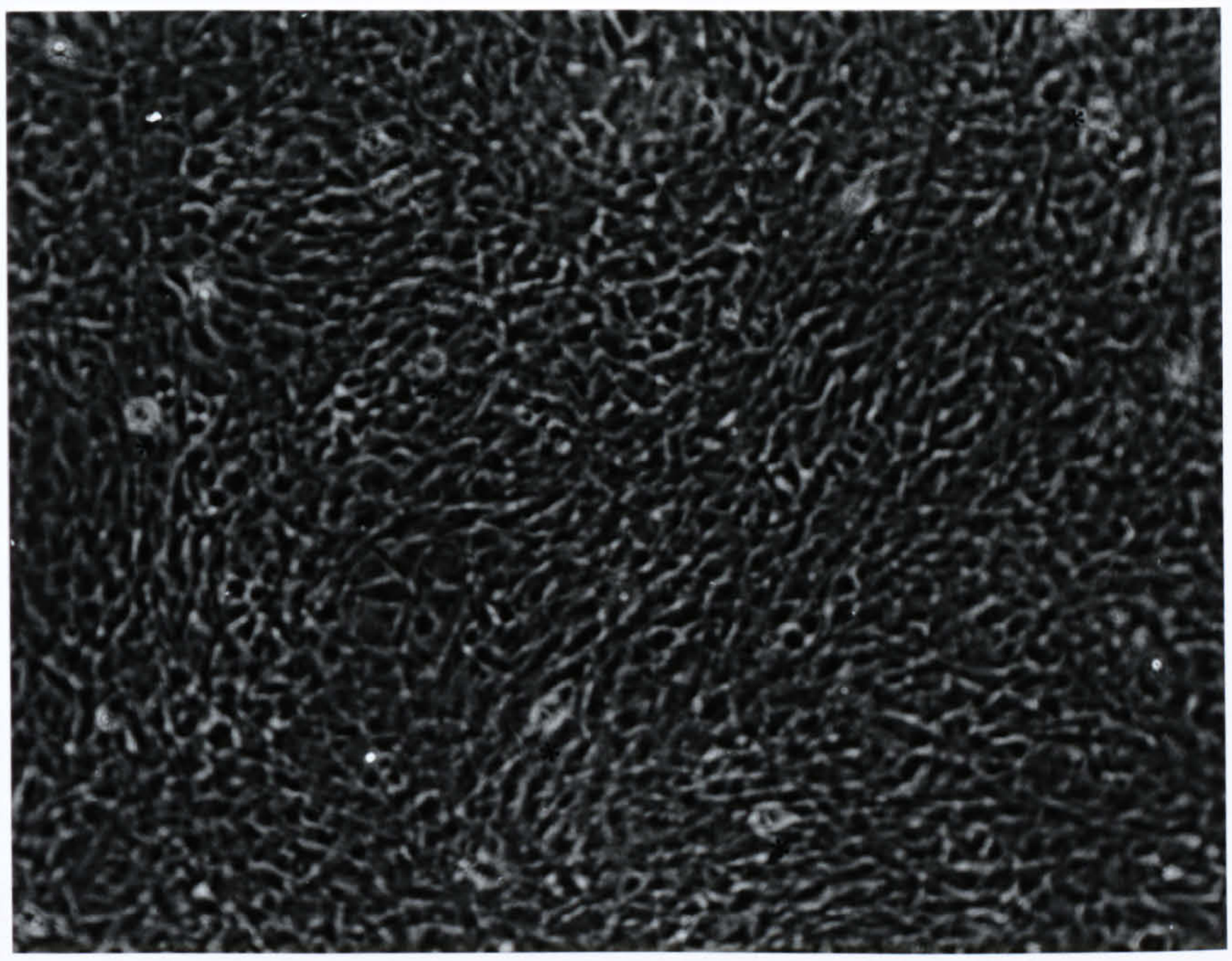


Figure 3.3. Growth in terms of total protein content of immortalised human osteoblasts (A) and rat immortalised osteoblasts (B) on the dense HA-Spinel material compared with cell culture on polystyrene culture plates. Culture were initiated at a seeding densities of $5x10^4/cm^2$ (THO) and $2.5x10^4/cm^2$ (FFC). Total proteins were measured by Lowry assay at different culture time. Results are mean +/- SD mean, n=4. *P<0.05, by ANOVA followed by Dunnett's test, comparing control and dense HA-Spinel data at the same times in culture.

(A)

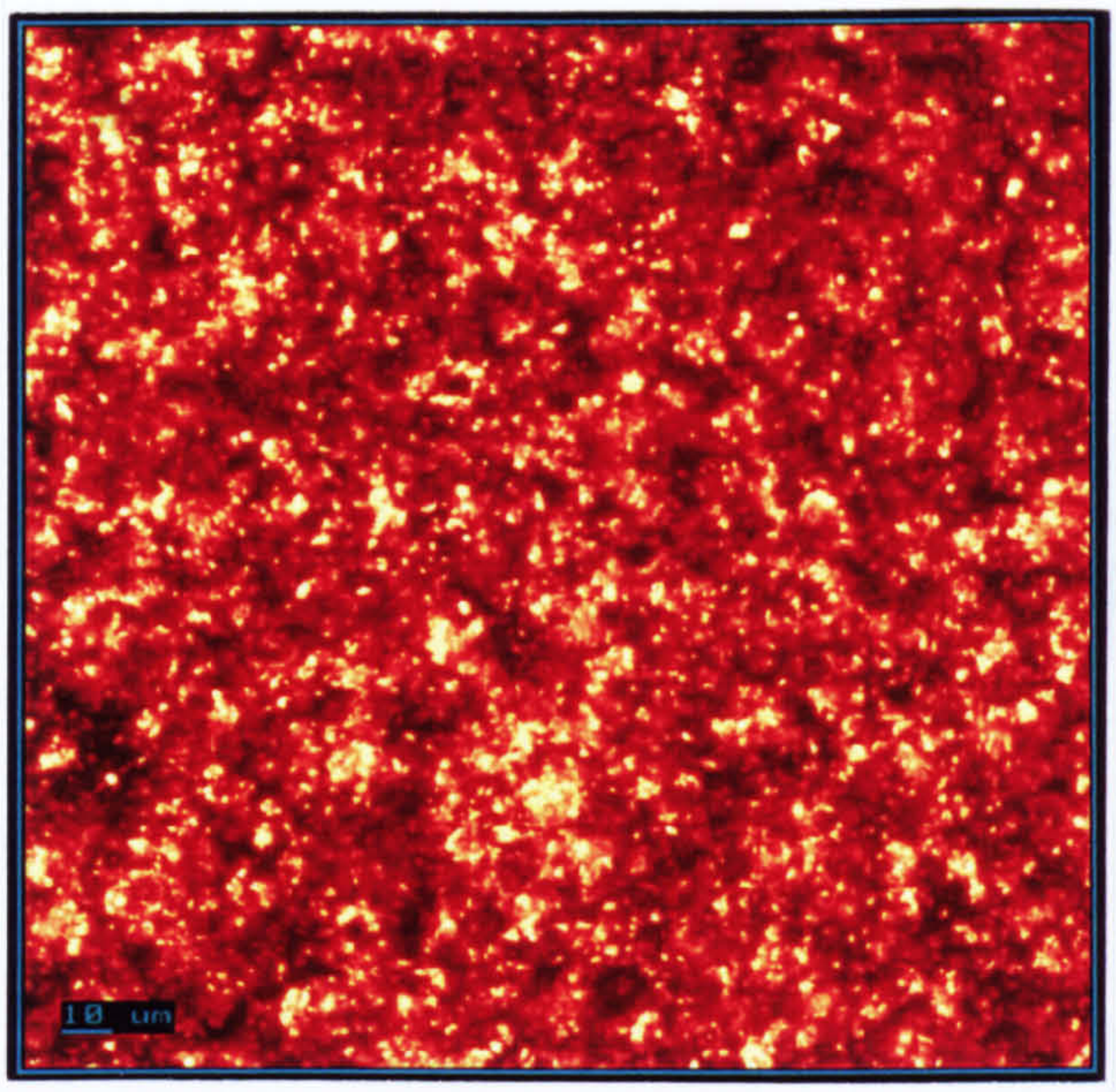


(B)

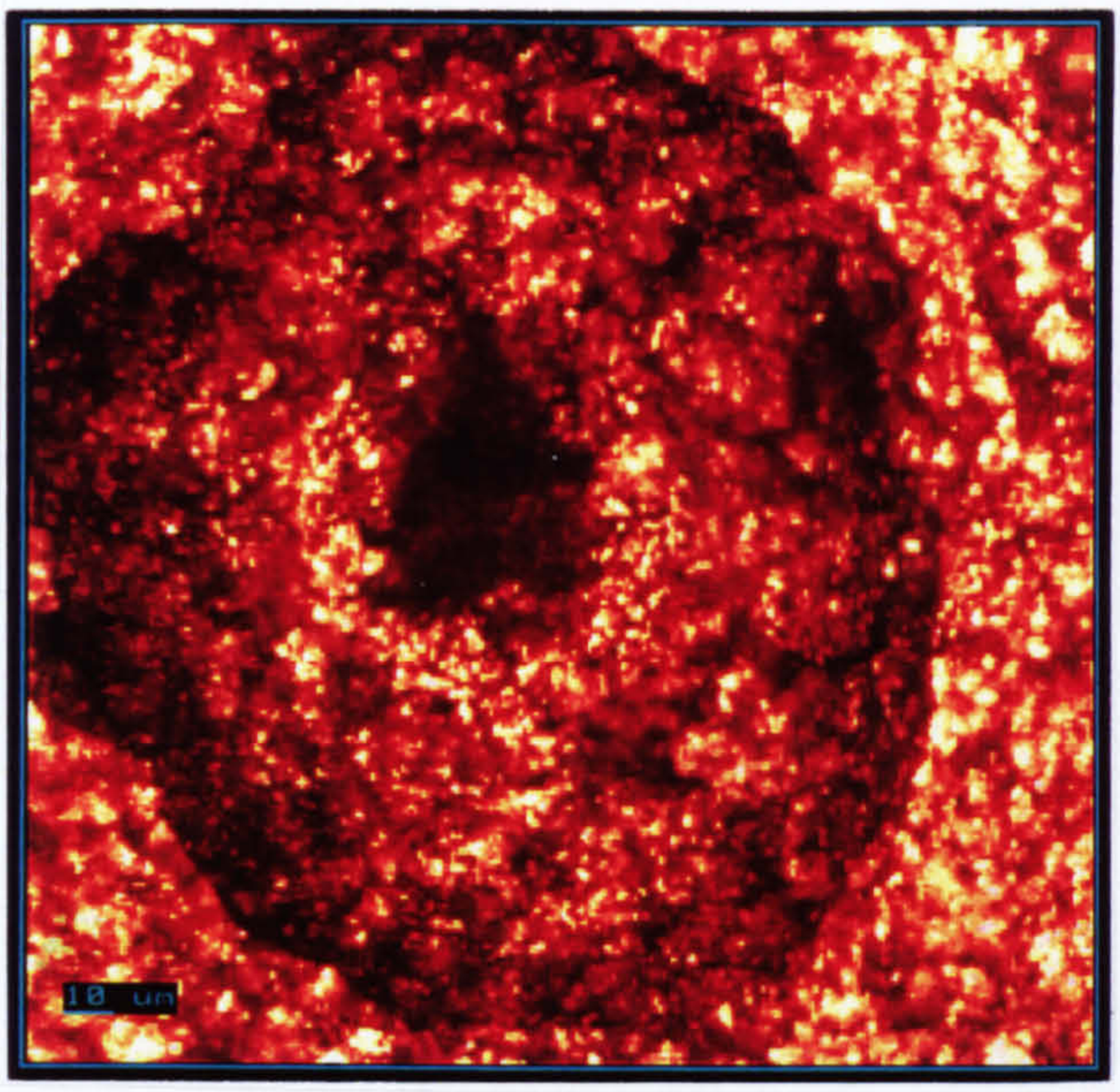


Photograph 3.1. Morphology of immortalised human osteoblasts (THO (A) and immortalised rat osteoblasts (FFC) (B). Cultures were initiated in standard 24-well plates (polystyrene, $2cm^2$ per well) at a seeding density $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells, incubating for five days and expressing normal morphology. Cell sizes range from 10-20 μm in diameter and * marked cells in mitosis. Magnification: x440

(A)

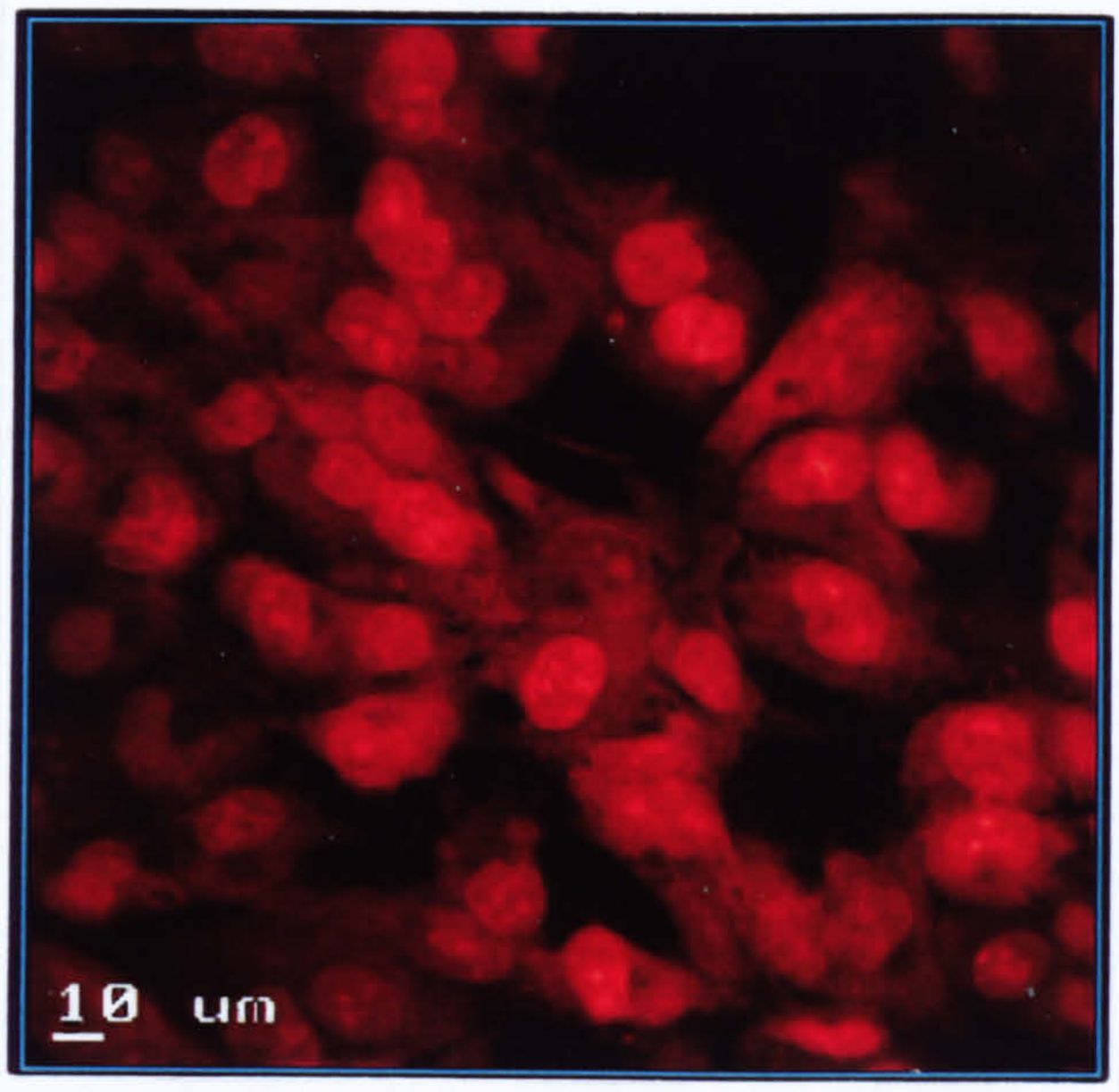


(B)

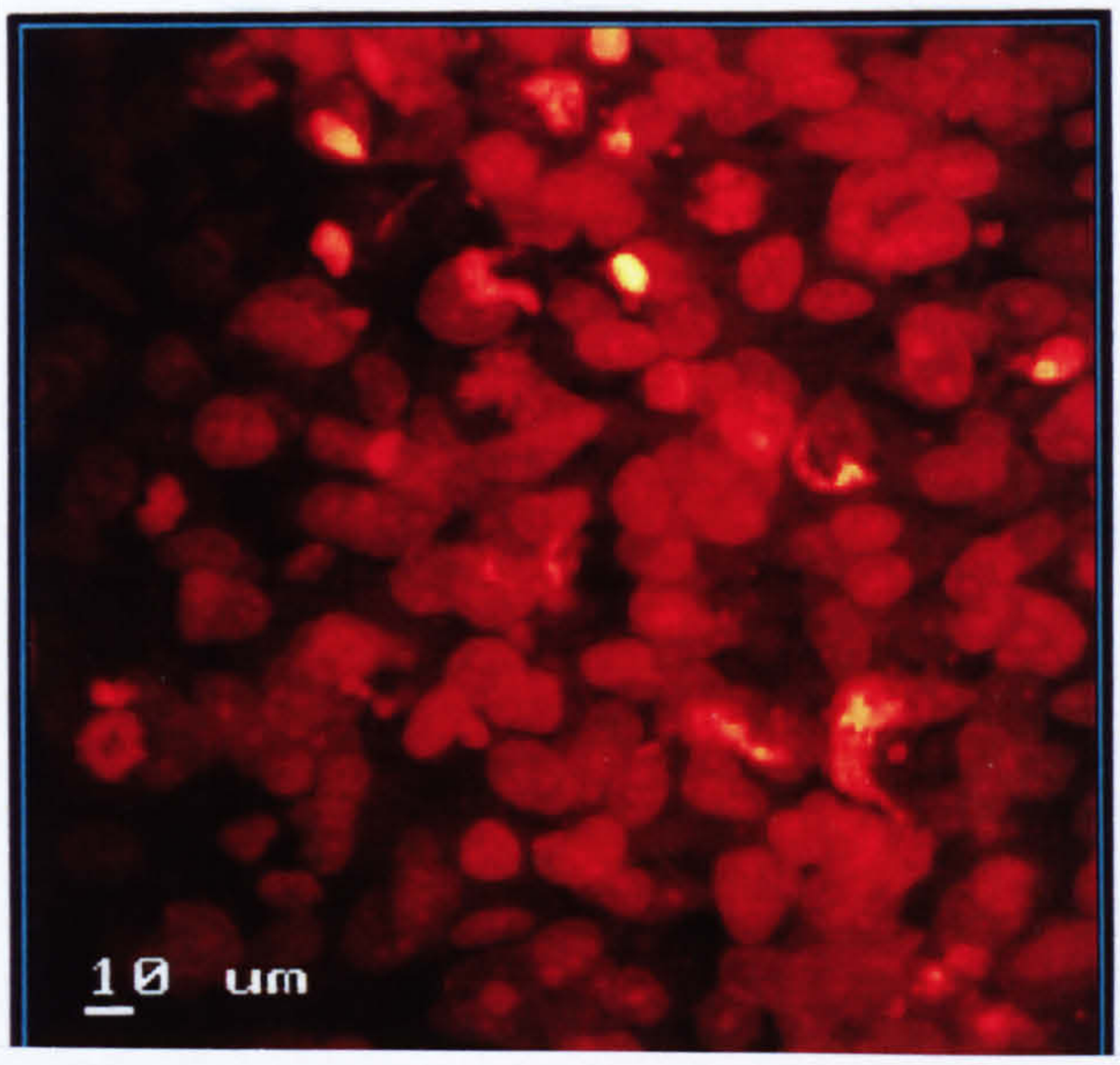


Photograph 3.2. CLSM reflectance images of the sand paper polished surfaces of (A) dense HA-Spinel material and (B) a pore in porous HA material. Scale bars are on the pictures and imaging area: 200 x 200μm

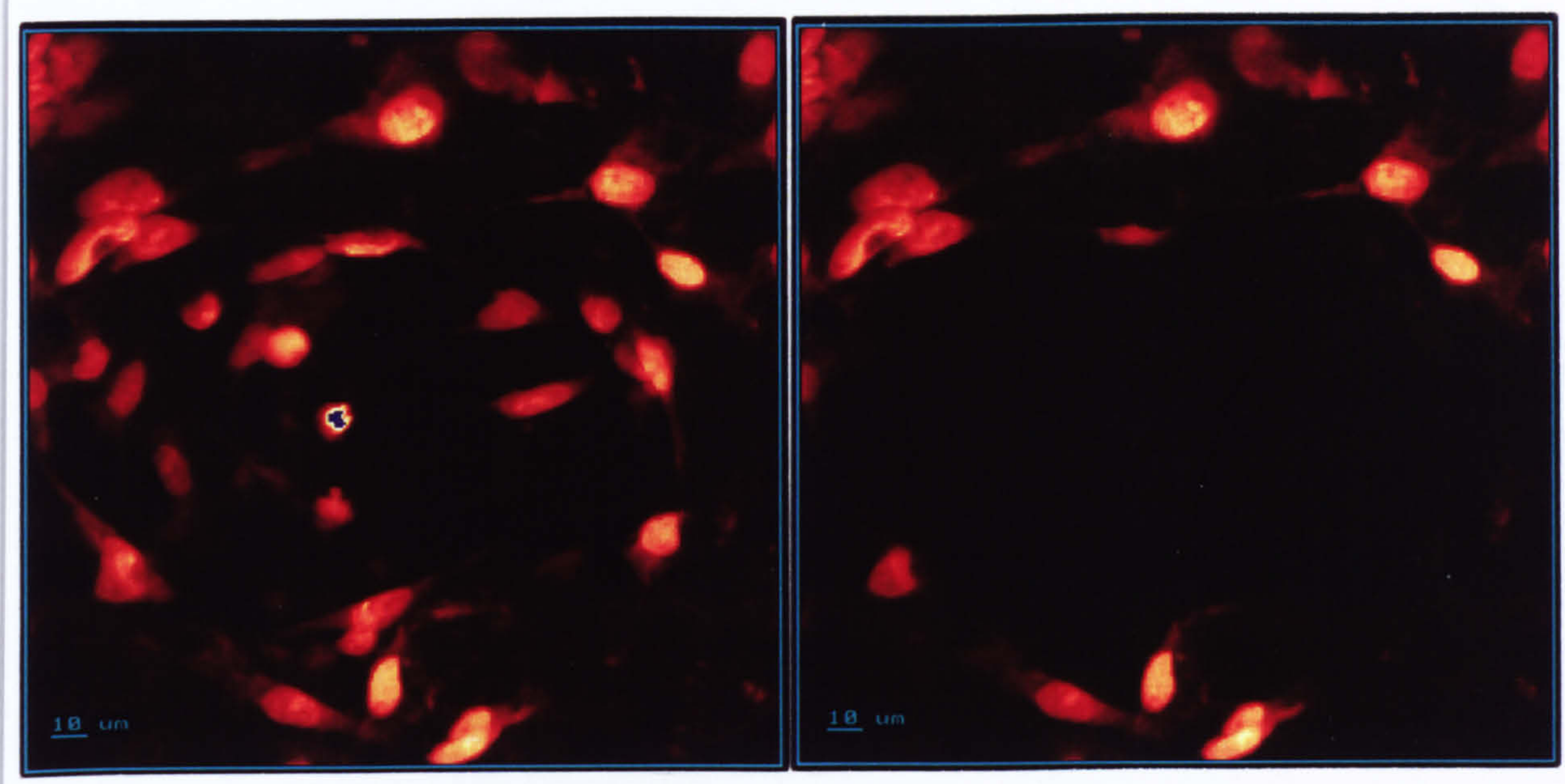
(A)



(B)

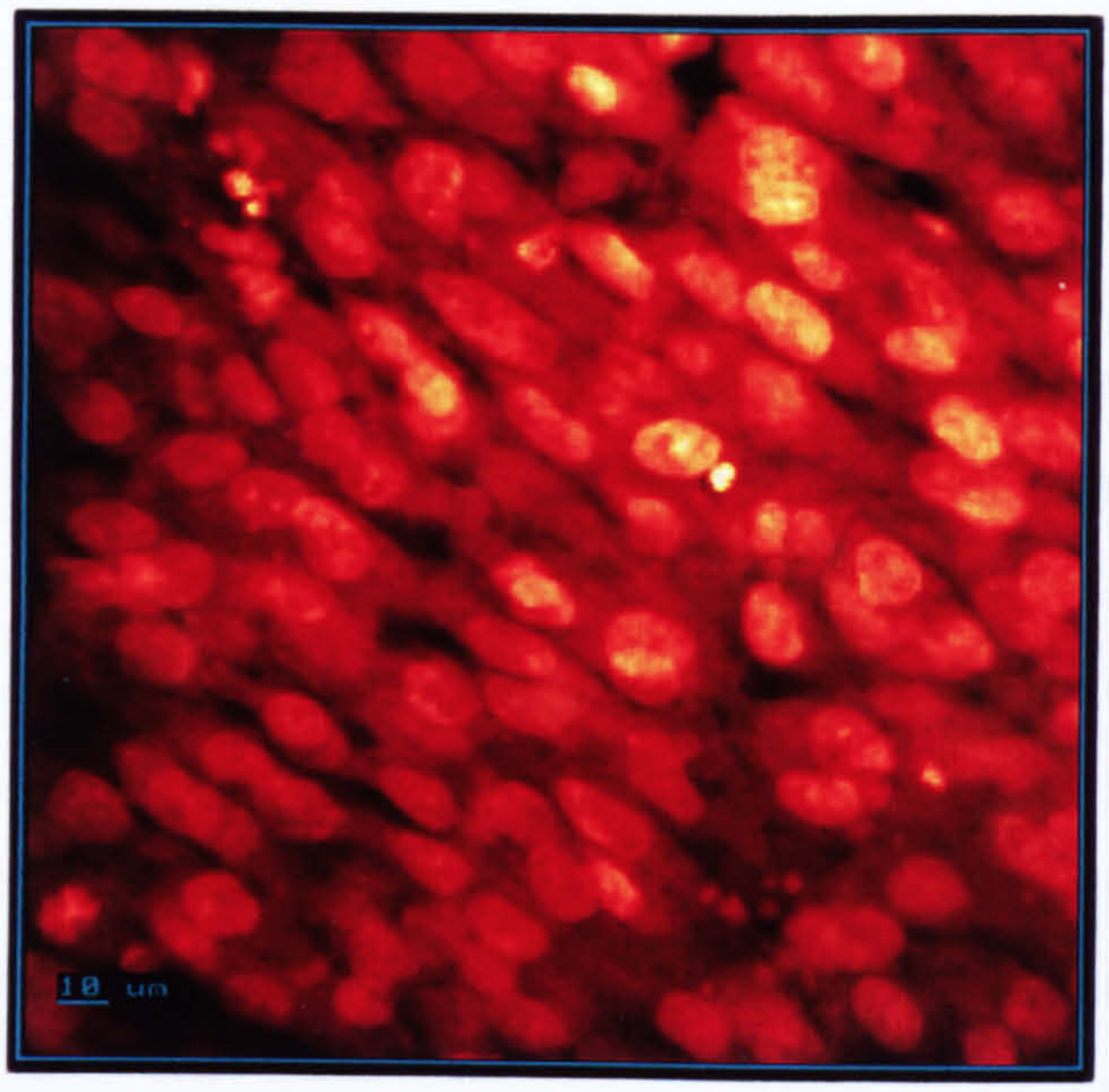


Photograph 3.3 (A) and (B). Distribution and morphological appearance of immortalised human osteoblasts (THO) after 4 days culture on the surface of dense HA-Spinel material. Original seeding density: (A) $2.5x10^4/cm^2$, and (B) $5x10^4/cm^2$. Cells were stained with 0.1% w/v ethidium bromide, followed by examination using CLSM with a x25/0.75 water immersion lens. Scale bars are on the pictures and imaging area: 200 x 200μm

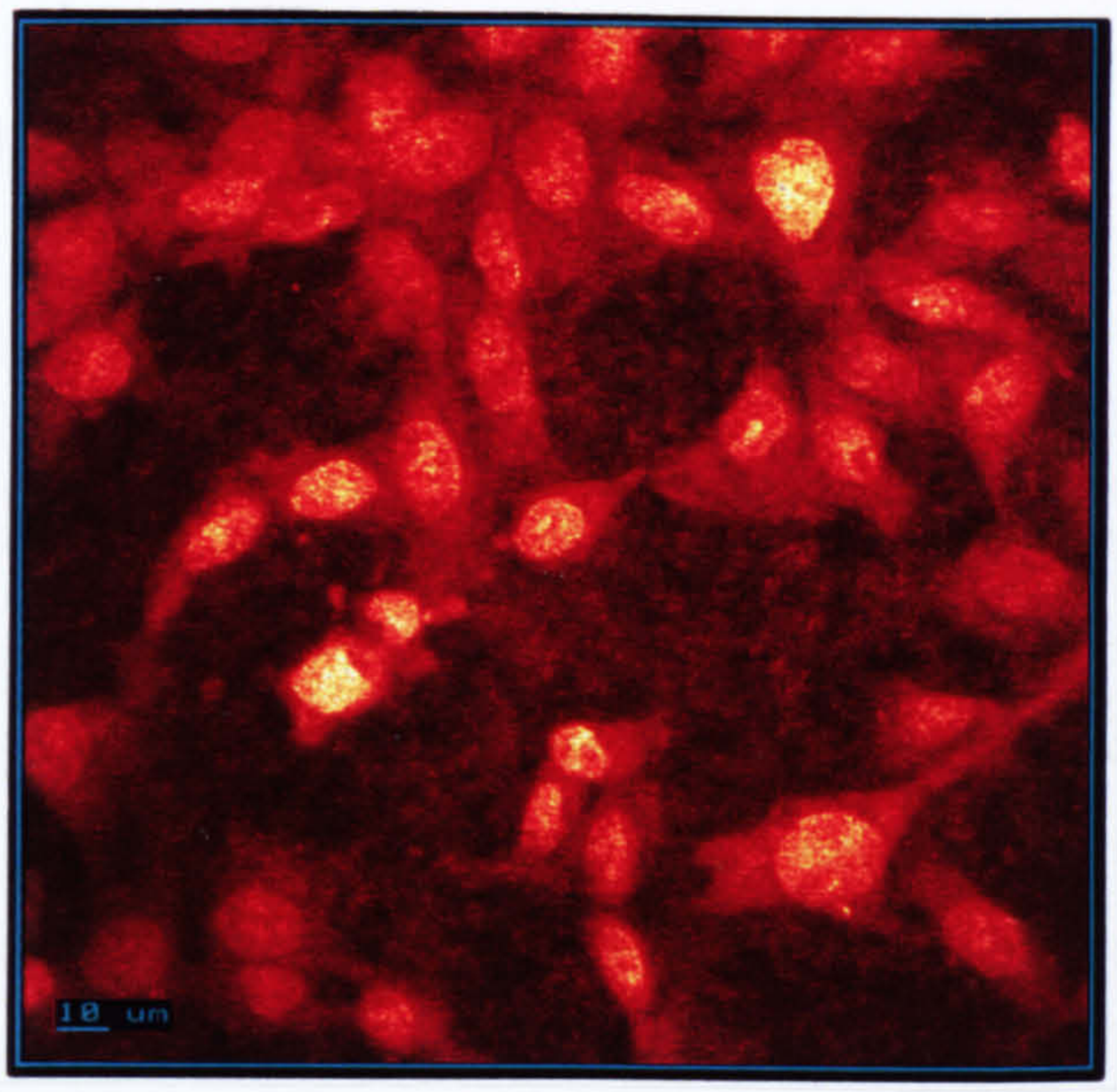


Photograph 3.4. Distribution and morphological appearance of immortalised human osteoblasts (THO) after 4 days culture on porous HA. Original seeding density: $2.5x10^4/cm^2$. Cells were stained with 0.1% w/v ethidium bromide, followed by examination using CLSM with a x25/0.75 water immersion lens. (A) is a stereo image of the stack of cells from surface to a depth of 130μm, and the remaining pictures are single layer images: (B) 20μm from surface; (C) 40μm from surface and (D) 130μm from surface (pore base). Scale bars are on the individual pictures and imaging area: 200 x 200μm

(A)



(B)



Photograph 3.5 Distribution and morphological appearance of immortalised rat osteoblasts (FFC) after 4 days culture on the surfaces of dense HA-Spinel material (A) and porous HA material (B). Original seeding density: $2.5x10^4/cm^2$. Cells were stained with 0.1% w/v ethidium bromide, followed by examination using CLSM with a 25x/0.75 water immersion lens. Scale bars are on the pictures and imaging area: 200 x 200μm.

3.4 Discussion

Dependent on the seeding density, the latent phase of cell growth may be longer or shorter. The growth curve of FFC in Figure **3.1** has demonstrated the influence of seeding density on the length of the latent phase. Compared with THO, FFC cells stop growing much more quickly. After latency, cells enter into a logarithmic growth phase and soon fill the area of the bottom of the flask. Due to contact inhibition (Adams, 1990; Zhen, 1995), cells stop growth (confluent phase). For THO cells, the passage should be made when the cells are less than 70% confluent, and FFC cells, when the cells are around 80-90% confluent. Experience has demonstrated that THO cell can be passaged 20 times and FFC cell can be passaged over 50 times. In the passage procedure we used trypsin solution to digest cell layer. Trypsin is a widely used digestion solution, and is used to peel cells off the substratum. Serum has the function to limit the action of trypsin. Therefore, after digestion, by directly adding serum, the activity of trace trypsin would be prevented by serum. During cell culture, the culture medium should be renewed every 48h to satisfy the nutrition requirement .

Initial interactions between a host and biomaterial by compounds originating from tissue are as yet largely unknown. Also cell attachment to materials surface is a complex procedure and most of it remains unknown. A hypothesis was proposed by Kasemo and Lausnaa (1991). Basically, the material surface is positively charged (surface chemistry of the material), and some parts of cell membrane are negatively charged. The surface bonding potential is determined by water molecules arriving at the material site within nanoseconds and bonding via hydrogen or oxygen atoms. The material composition and its preparation affects this first layer of water atoms, and hydrated ions. The excellent wettability of the material surface is useful to allow proteins to attach to the material surface, and subsequently, these proteins will mediate cell attachment to the material surface. The proteins involved in cell attachment and adhesion are discussed in section **1.8.** (pages 28-31).

Cell membranes could carry a negative charge called Zeta potential which results from the presence of carboxyl groups or other molecular charges. Material can therefore attach or repel cells through various electron charges and they may be attached closely to a positive surface, flattening their ventral surface for better apposition and mineralisation.

Hence, the substratum charge not only affects cell micromorphology but also the mode of adhesion and cellular synthetic activity. When discussing cellular response to the implant materials, it is important to understand the basis of cellular adhesion, and the factors which could influence cell growth and cell proliferation. A general expression is that cells do not bind to the material surface directly but bind to the excellular glycoproteins which are produced by the cells themselves (osteoblasts and fibroblasts) and adsorbed onto the material surface, such as fibronectin, vitronectin, osteopontin, etc. As there are numerous integrins on the cellular membrane, and different regions are specific for different RGD containing proteins, therefore, the protein on the material surface would mediate cells attachment. In my research the immersion of the materials in culture medium prior to seeding the cells is useful for promoting anchorage and attachment of cells onto the material surface. Experiments in the present research showed that both THO cells and FFC cells can anchor and firmly attach to the material surface within 60min and no detached cells were found in culture medium.

The biological characteristics of calcium phosphate based materials when used as implants depend on many factors such as material composition, microstructure, porosity, and pore structure (morphology and size). In the present study the good biocompatibility of HA and HA-Spinel materials has been demonstrated by in vitro cell growth on the material. Normal morphology of cells was retained after culture on both materials, and the cells were able to attach and grow on the material surface as well in the deep pores within the materials.

In clinical practice, for optimal osseointegration of the porous Ca-P based materials, the critical factor is that the composition should match the natural bone tissue, and in addition, the pore morphology must be suitable for ingrowth of soft tissue. The pore structure supplies a scaffold for the bone cells to climb and grow into. If the pore size is too small, it is difficult to induce tissue growth into the material, and therefore the rate of osseointegration and biodegradation will be limited. On the other hand, if the pore size is too big this also results in lack of integration of the materials. Animal experiments have demonstrated that eight weeks after the highly porous HA material had been implanted in the femur of rabbits, the living bone had grown into the deep pores of the materials (Weng et al, 1993). It is the first time that human osteoblasts have been observed in deep pores of highly porous HA materials in vitro by CLSM. Both

osteoclasts and osteoblasts may interact with porous HA in vivo and the consequences of the interaction with both cells will result in the porous HA being degraded or new bone forming. In other words, the degradation rate of the porous material and the rate of new bone formation depends on the interaction of osteoblasts and osteoclasts in vivo. In the author's previous work, by controlling the pore size and density, the highly porous HA material could be fully integrated into bone vacancies of human humerus and ilium (Ruan et al,1993). Based on the research results, we suggested that the porous structure of HA, when implanted into the human body, provides a three dimensional scaffold for osteoblasts to penetrate and colonise, facilitating the process of deposition of new bone. It also provides a three dimensional scaffold for osteoclast resorption. A difficulty in my research was that neither CLSM nor SEM are able to observe cells which had moved into the lateral pores within the material.

CLSM is an important method in many areas of biological and medical research. Detection, localisation, and even quantification of molecules in living cells are possible using this technique. By confocal scanning, the out-of-focus-blur can be eliminated. The illumination is restricted to a single point and almost all the light emission from regions above and below the focal plane of a CLSM if physically prevented. A clear image can be obtained (Sheppard and Shotton 1997). However, fading of fluorochromes during exciting is a unavoidable phenomenon, which is dependent on the excitation light and exposure time. This is a result of photo-chemical oxidation which leads to decomposition of the fluorochrome. To reduce this decrease in fluorescence, the specimen to be studied by CLSM is best stored in the dark at 4°C. Glycerol in fluorochromes can lengthen the life of the fluorescence emission(Lacey, 1989). The most effective way of reducing specimen fading is to minimise exposure of sensitive materials to incident radiation with respect to both intensity and time. Ethidium bromide and propidium iodide are frequently used to stain the nucleic acids in cells. They both show red fluorescence upon excitation with blue and green excitation light. Compared with SEM observation, CLSM is more favourable to observe cell in situ and to investigate the cell biological components and cell substructure by use of staining. There is no damage to cells during preparation of CLSM samples. The preparation involves simple processes and allows observation in three dimensions. Specially, by CLSM the cells on different layers behind the first layer can be clearly observed and their shape measured. Therefore, the cells attached to the

pore wall in the porous material could be scanned step by step down to the deep pore and numbers of cells at precise depths throughout the substratum could be estimated. Additionally, the cells could be in "wet" conditions when observed by CLSM because a laser is used in CLSM. A vacuum is not required. The most important disadvantage may be the limitation in magnification, hence the detail structure and detailed changes in cell can not be identified.

As a biomaterial, HA-Spinel composite was invented many years ago, and it has been used in the clinic, but the biocompatibility tests have only been made in vivo. In the present study, the osteoblast cells grown on HA-Spinel formed muticellular layers. Counting the cell distribution on both dense and porous HA biomaterials, there were 153 and 142 cells on the dense HA-Spinel and on the highly porous HA, respectively. In the present study the human osteoblast growth on the HA-Spinel dense biomaterial was quite normal, and was the same as the control group. These results are similar to those reported for MC3T3-E1 cells (osteoblast-like cells) on the porous HA material previously, but different from the reaction of MC3T3-E1 cell growth on porous bioactive glass (ElGhannam et al, 1997). In the latter case, the proliferation of MC3T3-E1 on the porous bioactive glass was significantly altered at different time intervals. Their results demonstrated that cell proliferation rate showed the peak point at day 7, then decreased notably.

Gomi et al (1993) demonstrated that osteoclast cells attached more easily to a smooth surface of sintered HA materials. In the present study it was found that after 4d of culture of human osteoblasts on both dense HA material and porous HA material, the cell numbers were similar (153 compared with 142). Perhaps by expanding the culture time, any differences in the ability of the materials to support cell growth would be detected, because the surface growing area of the porous material is much bigger than that of the dense material. In the next part of my research I have measured the function of the cells in terms of extracellular matrix synthesis on both HA-Spinel dense material and highly porous HA material.

Material surface condition is important for cellular adhesion and expression of functions as well as for morphological features. All of these could be significantly affected by surface structure and chemical composition of the implant materials. The mechanisms that are responsible for the effect of roughness of material surface on the cell function and

morphology are not clear. From the literature we can obtain contradictory views. For example, Bowers et al,(1992) demonstrated that significantly higher levels of cellular attachment (rat osteoblast like cells) were found on rough, sandblasted surfaces of titanium with irregular morphologies, and suggested that implants should be prepared with a roughened surface at the bony contact area. Meanwhile Kieswetter et al (1996) have obtained contrasting results when they cultured osteoblast-like cells on porous materials. However, in my research both human and rat osteoblasts can anchor and firmly attach to flat, smooth surfaces and rough surfaces, and can even attach to the pore wall in the porous material.

The results of cell culture on the materials have showed that with THO cells, there was a significant difference in total protein at day 7, and with FFC cells, there were significant differences at day 5 and day 6 when the cells were cultured on HA-Spinel. This implies that protein synthesis was limited on the HA-Spinel material. A similar phenomenon was found by Anselme et al (1996) and El-Ghannam (1997), who cultured human osteoblast-like cells on Ti alloy with HA coating. One possible explanation for the decrease in cell number is that the material may induce programmed cell death (El-Ghannam et al, 1997). Figure **3.3** shows that apart from those time points previously mentioned, in general, cell growth behaviour on the HA-Spinel materials is similar to that of cell culture on polystyrene culture plate and the total protein synthesised on HA-Spinel materials is close to that on polystyrene culture plate. It is suggested that these bio compatible results are related to immersion treatment in culture medium prior to cell seeding on the material discs. I immersed the samples for 48 h in my experiments. The immersion time is commonly chosen between hours to seven days (Steele et al, 1992; Lu et al, 1996, Akazawa and Kobayashi, 1996), and as had been reported earlier, immersion in culture medium up to 48 hours promotes the formation of an amorphous calcium phosphate rich layer on the glass surface (Ducheyne and Shapiro, 1996)

A major problem with cell culture models is that normal bone cells in culture show alteration of phenotypic properties(de-differentiation) with increasing passage number (Zhen, 1997). They finally lost their proliferation activity. For example, in the present research, the THO cells lost their proliferative capacity at the 20th passage. I failed to culture THO cells at low seeding density on the porous materials, if the material had not been immersed in culture medium. I believe that there are two factors which

produce this effect. One is from the cells themselves. For cells to grow they need to communicate with each other, and at low density due to lack of communication, cell colonisation in the deep pores would be impaired. Another factor is from the material surface. In accordance with the phenomena of acute inflammation observed when the porous materials are used in the clinic, there is perhaps, a toxic reagent present in the material. This consideration will be discussed in next chapter.

Chapter 4 Indirect cytotoxicity evaluation of calcium phosphate biomaterials in vitro

4.1 Introduction

Dissolution and degradation of implant materials in body fluids may lead to local inflammation, system toxicity, or allergic reaction (Ferguson et al, 1960; Park and Lakes, 1992; Pelletier and Druet, 1995). It is important therefore to evaluate the toxicity of the implant materials.

Biocompatibility assessment of implant materials is based on in vitro and in vivo approaches (Williams, 1981; Prigent et al, 1998). Evaluation of tissue responses when in contact with the biomaterial itself, its released substances and soluble or insoluble degradation products, is carried out in vitro. If no overt toxicity is recognised, the material could be then implanted into host tissue and the response examined in vivo. Extrapolation is carried out from qualitative and quantitative data, obtained in vitro from the interface of the potential implant and cell system, representative of the tissue which it will confront in vivo. The toxicity assessment involves indirect and direct cytotoxicity (Paul, 1979; Bruner et al, 1991; Burton et al, 1986; Hunt and Williams, 1995;). Indirect cytotoxicity is based on assessing the toxicity of soluble material extracts which are made by immersing the test materials in culture medium, then seeding and culturing cells in the extracts. Subsequently, the effect of the extracts on the cell morphology and other biological features, for example cell growth and cell proliferation, and enzyme activities are examined. If the culture medium was used as an immersion liquid, the leachable compounds from the material into the immersion medium could be influenced by the serum in the medium. Two general approaches to studying the in vitro toxicity aspects of implant materials can be considered. These are: 1. in vitro tests on aqueous extracts; 2. in vitro direct tests on the materials. Tests on the aqueous extracts, i.e. indirect cytotoxicity tests, can provide rapid and quantitative results, and their main purpose is to screen potential biomaterials for this next stage analysis. The main advantages of indirect cytotoxicity tests, for example, the MTT assay, are that they can be performed on cells grown in microtitre plates using an automatic microplate reader. This offers considerable saving in time while being simple and convenient. Indirect cytotoxicity assays do not allow prediction of the interaction between cells and material while in direct contact. The

direct tests in vitro can provide this information relating to the cell/biomaterial interface such as the degree of cell spreading, cell adhesion, the effect of surface condition on cell morphology, and the evaluation of collagen synthesis on the biomaterials.

Since the 1980s a lot of cases dealing with successful or unsuccessful applications in clinic have been reported. Successful programmes usually resulted from the excellent biocompatibility and boneconducting ability of the biomaterials (Caulier et al, 1995; Bucholz et al, 1993; Masaki, 1995). On the other hand, unsuccessful programmes often demonstrated that host organs or tissues excluded orthopaedic implants and therefore local inflammation took place (van Blitterswijk et al, 1995; Nilsson et al, 1994; Biasini et al, 1992). Exclusion and inflammation in the clinic are observed for various reasons including material chemical composition, physical properties as well as macro- and micro-structure of the implants, surface condition of implants, and also patient dependent factors. Nevertheless, it must not be overlooked that no matter what kinds of materials are used in orthopaedic application, it is necessary for the mineral apatite or apatite carbonate layer to form a bond at the tissue interface (Kotani et al, 1991; Akao et al, 1993). For example, the knee and hip joints made from metals and alloys usually lack calcium phosphate cover, so that the toxic actions of the metallic ions leaching from the implant, and the corrosion of the implant by biological liquids are a continuous threat. A fibrous capsule is formed surrounding the implant, which isolates the implants, limiting the toxic effects of the material and the corrosive action of the biological environment. Calcium phosphate materials with improved properties, and orthopaedic implants coated with calcium phosphate will be potential candidates for future development (Klein et al, 1994; Wilson et al, 1995; Cameron, 1994).

Since there are so many factors responsible for rejection and inflammation, it is difficult to understand the contribution produced by each of the factors in vivo. However, the interaction between implant materials and cells can be investigated more readily in vitro. The object of this study was to investigate the toxic response of an immortalised human osteoblast cell line (THO) and immortalised rat osteoblast cell line (FFC) to HA-Spinel using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay, reduced glutathione (GSH) determination, and Lowry assay for total protein, to reveal the early stage of the interactions of biomaterials and cells.

The MTT assay is used to test chemicals that elicit toxicity because of the advantages offered in terms of speed and convenience. By MTT determination, the indirect toxic response can be directly read in cells growing in extracts of materials in microtitre wells using an automatic micro-plate reader (Sagouras and Duncan, 1990; Marshall et al, 1995). The basic biochemical process is that a tetrazolium salt is added to the wells containing cells and after incubation to allow formazan production, a direct relationship is established between increased coloured formazan production and the number of cells in each well. Any increase in formazan production is then attributed to an increase in cell number. The basic principle of MTT assay is a spectrophometric determination which detects the conversion of tetrazolium salt MTT by dehydrogenases present in viable cells into a insoluble formazan product. The MTT assay is used extensively to test chemicals that elicit toxicity and the tetrazolium salts in common use today are relatively stable and water-soluble and vary in colour from white to yellow. Their most important characteristics is that after reduction inside viable cells, the formazans which they produce are highly coloured and accumulate inside the viable cells. However, under certain conditions they cannot distinguish between cell loss (death) and metabolic inhibition, and the assessment quality is affected by the difficulty in dissolving the formazan reaction product. Cell density, pH and MTT concentration affect the absorption spectrum (Plumb et al; Jabbar et al, 1989; Bisset, 1995).

Intracellular GSH content of cells is a sensitive indicator of toxicity due to both electrophilic species and to oxidation stress (Macnair et al, 1997). GSH is one of most important factors in cellular defence against toxic materials. It is present in high concentration in most living cells and almost entirely in its reduced form (Walum et al, 1990). One of the functions of GSH is to detoxify reactive intermediates formed intracellularly. These intermediates include peroxides and free radicals which may be produced from xenobiotics either spontaneously or through metabolic process. GSH acts as a reductant and is oxidised in the process to its disulphide (GSSG) (Walum et al, 1990). Depletion of intracellular GSH is known to be one of the most critical effects of toxic injury of cells, since loss of GSH protection against reactive metabolites rapidly leads to cell death (Hissin and Hilf, 1976).

Lowry assay detects the total protein content of cell culture at different time intervals (as discussed in Chapter **3**) and basically reflects the effects of material extracts

and the materials themselves on the cell protein synthesis (Lowry et al, 1951; Darbre, 1986; Macnair et al, 1997). Those methods have been extensively used to detect the toxic responses of cells to drugs and chemicals. Also via culture of cells directly on the material, other features including phenotypic expression and normal function of cells can reveal the response of the cell to the implant material in vitro. The synthesis of collagen was measured by the incorporation of tritiated proline into the protein. This method was chosen because it is readily quantified, so could reveal the effects of the material extracts on the process. There are other methods for detecting collagen synthesis, and these are discussed in more detail in Chapter **5**, but this method provided a convenient means of comparing the influence of the extracts on the major function of the osteoblasts. Incorporation of ^{3}H-proline probably only gives an indication of the rate of production of the pro-collagen molecule, and it must be borne in mind that this undergoes considerable post-translation processing before final secretion, followed by cross linking of the collagen fibres. Measurement of ^{3}H-proline incorporation does not necessarily reflect production of extracellular collagen fibres; this is measured using immunostaining techniques in Chapter **5**.

In order to understand the tissue interactions during the early period of implantation of hydroxyapatite and related biomaterials, this chapter will discuss indirect toxic responses of human and rat osteoblast cells to material extracts and to the HA-Spinel material itself. Protein determination by the Lowry Assay is equivalent to cell proliferation as discussed in Chapter **3**. GSH content reveals mechanistic information on toxicity, and the MTT assay indicates cell viability. In addition, the specialised function of collagen synthesis of the cells when cultured in the material extracts was quantified.

4.2 Methods and Materials

4.2.1 Materials and extracts

Dense HA and HA-Spinel biomaterial was manufactured as described in Chapter **2.2**. The cytotoxicity of the HA and HA-Spinel biomaterials was assessed by preparing aqueous extracts according to the recommended method of International Standards Organisation (ISO) 10993-3, 1992 (British Standards and European Norme (BSEN) 30993-5,1994). The tissue culture plastic polystyrene was used as non-toxic negative control material and tin-stabilised (tributyltin) poly vinylchloride (Portex Ltd., Kent) was used as the positive toxic control material. The positive control material was measured as discs of 12mm diameter and 2 mm thickness, and the HA and HA-Spinel as discs of 12mm diameter and 3mm thickness as described before. Before preparing the aqueous extracts, the HA and HA-Spinel discs were sterilised in the autoclave. The control material discs were immersed in 70% (v/v) ethanol for 1 hour prior to treatment with PBS containing 100 units/ml penicillin and 100μg/ml streptomycin for 1 hour. Then all the discs were immersed in the extracting media for preparation of the extracts, according to the recommended method of ISO 10993-3, 1992 (BSEN 30993-5, 1994). The extract media were DMEM supplemented with 10%(v/v) FCS, 50 IU/ml penicillin and 50μg/ml streptomycin for FFC cells and Ham's F10 containing 10%(v/v) FCS and the antibiotics as same above for THO cells. The ratio of the volume of the extractant to the surface area of the material was 1ml/cm^2. The extraction process was carried in a water bath at 37°C in 75 cm^2 flasks which were shaken at a shaking speed of 60~65 returns/min. The extracting time was 48 hours, and at the end of 48 hours the extracts were passed through a 0.22 μm filter, and stored at -20°C. In order to investigate the effects of immersing for 48 hours in media on the material surface, we prepared a second extract by using the same discs and the same method. Effects of this second extract on the cells was then investigated. The reason for making the second extract using the same material is to understand the influence of the immersion on the material surface, composition and cytotoxicity.

4.2.2 Measurements of indirect cytotoxicity

4.2.2.1 GSH assay

For GSH determination of the extracts, both THO at passage 12 and FFC at the passage 32 were cultured in 24-well plates and the seeding density of THO cells was $1x10^5$ cells per well and the seeding density of FFC cell was $5x10^4$ cells per well. Incubation conditions were same as the described in Section 2.2. Two hours after seeding, the normal medium was removed and 1ml of extracts added to each well. Thereafter the extracts were renewed every two days. At 24 hr intervals, the extracts were aspirated and the cells were washed with PBS. The intracellular GSH was extracted with 200 µl aliquots of 10% Trichloroacetic acid (TCA) at room temperature for 10 minutes. Then the GSH extracts were collected and stored at -20° C. Fluorimetric analysis of GSH content in extracts was measured on a spectrofluorimeter (Shimadazu RF-5001 PC). GSH was determined by the method of Hissin and Hilf (1976). Cell debris were centrifuged out of the extracts by spinning for 3-5min at 13000g in a microcentrifuge. Twenty five microliter extract was mixed with 2.3 ml of phosphate buffer, pH8 (Composition is given in appendices), and 100 µl of 1mg/1ml O-phthaldehyde (OPT) in ethanol. The mixed solutions were left at room temperature in the dark for 15min. Standards were prepared with a concentration range of 0~50 µM GSH, and the data were read at wavelengths of 350nm excitation and 420nm emission, using water to zero the fluorimeter.

4.2.2.2 Total protein assay

With total protein assay, after TCA was aspirated from the wells, 1 ml 0.5 M NaOH solution was added to each well and the plates were sealed with clingfilm and incubated at 37° C. Eighteen hours later, the NaOH digested THO and FFC cells were aspirated from the wells, and stored at 4° C for total protein concentration determination by Lowry assay on a UV-2101 PC Scanning Spectrophotometer. Total protein of both THO cells and FFC cells cultured in extracts were measured by the Lowry assay which was described in Section 3.3

4.2.2.3 MTT assay method

MTT assay was directly carried out on cells grown on 96-well plates. Cells were cultured in 96-well microplates at a seeding density of $5x10^4$/per well for THO cells and

$2.5x10^4$/per well for FFC cells. Twenty four hours later the culture media were replaced by 200 µl of the extracts added to each well and the cells incubated for another 48 hr. After this time the extracts were aspirated from the wells and 50 µl of 10 mM MTT in PBS(pH 6.75) was added to each well. After incubation for 4 hr the liquid was aspirated again and the MTT formazan reaction product dissolved in 200 µl dimethyl sulphoxide (DMSO). The optical densities were measured on a Biorad Model 450 microplate reader at 540nm.

4.2.3 Measurement of Collagen synthesis

For the measurement of collagen synthesis function of cells cultured in extracts, FFC and THO cells were seeded in 24 well plates at a density of $2.5x10^4$/well and $5x10^4$/well respectively, and 2 hr later, the normal culture media were replaced by appropriate extracts. 48 hr later, cells were treated with 37 KBq/ml L-(2, 3-^{3}H)-Proline, specific activity 1.4 TBq/mmol (Amersham), in the appropriate extracts (1ml). After exposure to the radioactive label reagent for 48 hours, the cells were washed with PBS 3 times (about 10min for each wash). One millilitre 1mM proline in isotonic PBS was added to each well, and cells incubated for 10min at room temperature to remove the non-specific ^{3}H-proline bound inside the cells. Cells were washed with PBS again and were extracted in 2ml 0.5M acetic acid, overnight at room temperature. For each sample, a 500 µl aliquot of the extract was neutralised by 0.5ml 0.5M NaOH and 1mg (404 Units) of type IV collagenase (Sigma) in Hank's II buffer (stock solution 100mg type IV collagenase in 2ml Hank II, the composition of Hank's II buffer is in appendix I) was incubated with it for 60 min at 37°C. Noncollagen protein (NCP) was precipitated by adding 100 µl 10% TCA at 4°C for 15min followed by centrifugation at 13000 rpm for 10min in a Micro Centaur (MSE). Supernatant (0.5ml) containing collagenous protein (CP) was transferred to a vial containing 4 ml scintillation fluid (Hionic-Fluor, Packard). Radioactivity in the form of disintegration per min (dpm) was determined by a Packard Tri-Carb 1900 TR liquid scintillation analyser by using quench correction by TSIE/AEC. Meanwhile, 100µl of the acetic acid extracts of each sample was transferred to a vial containing 4 ml scintillation fluid and radioactivity in the form of disintegration per minute (dpm) was measured. As the latter included both collagen protein and non-

collagen protein, by comparison of the radioactivity incorporated into collagen protein with that in collagen protein plus non-collagen protein, the percentage of collagen protein in the total protein was readily estimated.

4.2.4 Statistics

All measurements were collected and expressed as means +/- standard deviations. Single factor analysis of variation [ANOVA] followed by Dunnett's test was employed to assess the statistical significance of results for all the experiment data at the 95% confidence limit (<0.05).

4.3. Results

4.3.1. Morphological observation

Photograph **4.1-4.4** shows the morphological observation of THO and FFC cells cultured in the extracts. Both THO and FFC cells appeared to show normal morphology in the control **(Photograph 4.1**: A, B) and first HA extracts **(Photograph 4.2**: A, B). THO cells also appeared to show normal morphology in the first HA-Spinel extracts (**Photograph 4.3**: A), but the cell number was decreased. Although FFC cells in the first extracts appeared to have normal morphology, the number of the cells was notably decreased **(Photograph 4.3**; B). This indicated that FFC cells were more sensitive to the effects of the HA-Spinel extracts and the extracts exerted an effect on FFC cell growth. After 48 hours exposure to the positive control material extracts, almost all of the FFC cells died and detached off the bottom of the culture plate. In the positive control extract few of the THO cells were still attached to the culture plate, but the morphology appeared abnormal(**Photograph 4.4**: A, B). These results shown that a slight toxicity was present in HA-Spinel materials, but it was not as toxic as the positive control extract. The positive control was severely toxic and caused a marked de-attachment and death in cell culture. Photograph **4.5** shows the SEM micrographs of the deposits on the surface of HA-Spinel discs which had been immersed in DMEM culture medium for 48 hours. The structures and compositions of the tiny particles with different morphologies deposited on the material surface were not detected. These white particles showed up as different from the dark background of the substrate material HA-Spinel, and some of them were shaped like crystals with size 0.1-1μm.

4.3.2. GSH measurements

Figure **4.1** shows the response of THO (A) and FFC (B) cells to extracts of the HA-Spinel. FFC cells appear to be more sensitive to the depletion of GSH both from the HA-Spinel and the positive control extracts; this is possibly because they are a faster growing cell line. With the THO cells, the effect of the first extract was not apparent until after 3 days in culture. After this time, the first extract depleted GSH to 72.3+/-6.9% of that measured in control cells at day 3 and 81.6+/-4.1% at day 4. The result at day 3 was

statistically different from controls. In contrast to the effect of the first extract, the second extract increased GSH level at all time points in THO cells culture. With THO cells in control group, the GSH contents were 3.1+/-0.49 nmol per well at day 1, 3.2+/-0.27 nmol per well at day 2, 4.2+/-0.66 nmol per well at day 3, and 3.8+/-0.77 nmol per well at day 4. Correspondingly, with THO cell in the second extract, the GSH contents were 3.75+/-0.54, 4.4+/-0.37, 4.90+/-0.56 and 4.7+/-0.74 nmol per well at day 1, day 2, day 3, and day 4. Comparing these data in the two groups, the GSH contents in second extract were slightly higher that in control group and except for the data on day 2, there were no significant differences.

With FFC cells, GSH contents in second extract were 1.0+/-0.27 nmol per well at day 1, 2.7+/-0.41 nmol per well at day 2, 7.3+/-0.71 nmol per well at day 3, and 6.8+/-0.22 nmol per well at day 4. Correspondingly, GSH contents in control group were 0.8+/-0.31, 2.9+/-0.59, 6.3+/-0.48, and 7.8+/-1.0 nmol per well at day 1, day 2, day 3, and day 4, respectively. There were no statistically significant differences between the above two groups. FFC cells responded to first extract by a depletion of GSH which was apparent at all time points and the data were 0.4+/-0.1, 0.3+/-0.16, 3.6+/-0.45, and 4.1+/-0.39 nmol per well at day 1, day 2, day 3, and day 4, respectively.

To facilitate comparison of the responses of THO and FFC cells to the extracts, the GSH content of both cell types is shown on Figure **4.2** expressed as percentage of the control GSH values at each time point. This emphasises the greater sensitivity of the FFC cells to the GSH depleting effects of the extracts. Although the first extract of HA-Spinel caused marked depletion of GSH, particularly to FFC cells, it was not as toxic as the positive control extract. The positive control extracts caused a marked, significant decline in GSH levels to 48.3+/-11.87(highest) and 22.0+/-7.77 (lowest) of the control values at all time points for THO cells and to 56.4+/-22.95 (highest) and 1.7+/-3.10(lowest) of the control values at all time points for FFC cells. GSH contents of FFC cells in the first extract were decreased to13.2+2.0 (lowest) of the control value at day 2 and 57.8+/-5.94 (highest) of the control value at day 3. These were statistically different from control. The second extract did not cause a significant alteration in GSH levels over the time period of exposure investigated. This implies that the toxic factor involved in the depletion of GSH by HA-Spinel may be removed by the first extraction in medium for 48 hours.

4.3.3. Total protein assay

Figure **4.3** shows the total protein assay of the THO (A) and FFC (B) cells compared with the control group. Both THO and FFC cells are sensitive to a reduction in total protein caused by exposure to both HA-Spinel and positive extracts, but similar to the depletion of GSH, the FFC cells appear to be more sensitive. With both THO and FFC cells, the effects of the first extract were not apparent until after 3 days in culture. With the THO cells, after this time, the first extract decreased total protein to 69.7+/-11.45% of that measured in the control cells at day 3 and 72.0+/-2.74% at day 4. The results at day 4 were statistically different from controls ($p<0.05$). With the FFC cells, the first extract decreased total protein to 38.8+/-9.17% of that measured in the control cells at day 3 and 47.8+/-7.22% at day 4. Both of these results were also statistically different from control ($p<0.05$). Compared with the effects of the first extract, the second extract does not cause a significant alteration in total protein levels over the time period of exposure investigated. With THO cells, total protein produced in second extract was higher than that in the control group at day 1 (131.2+/-12%), day 2 (153.3+/-15.1%), and day 4 (147.6+/-13.6%), taking the total protein in control group as 100% at all time points. Although the total protein produced by FFC cells in second extract was lower than that in control group, 92.5+/-5.2% at day 1, 97.7+/-12.7% at day 2, 91.0+/-6.9% at day 3, and 96.8+/-4.9 at day 4, there were no statistical differences. Similar to the situation with GSH depletion, with the THO cells, the positive control extracts caused a marked, significant decline in total protein levels to 56.8+/-3.18% (highest) of the control values at day 1 and to 27.4+/-1.55% (lowest) of the control values at day 4, and with the FFC cells to18.8+/-7.77% (highest) at day 1 and to 6.53+/-3.19% (lowest) at day 4. The results obtained imply that the toxic component involved in the decrease in total protein by HA-Spinel is removed by the first extraction in medium for 48 hours. Figure **4.4** shows the comparison of the response of THO and FFC cells to the extracts according to the total protein levels, where the data have been expressed as a percentage of the control total protein values at each time point. The first extract of HA-Spinel caused a marked decrease of total protein, particularly to FFC cells, but it was not as toxic as the positive control extract. A strange phenomenon was that the total protein synthesised by FFC cells increased in the first extract at day 1. This may represent the cells attempting to defend themselves from toxicity by switching on protein synthesis.

4.3.4. MTT assay

Figure **4.5** shows the MTT assay of the THO (A) and FFC (B) cells cultured in the extracts of the HA-Spinel. The number marked on the Y-axis is the relative absorbance, and the data have been expressed as a percentage of the control absorbance. In contrast to the effect of the first extract on the depletion of GSH and total protein, THO cells appear to more sensitive than FFC cells to the HA-Spinel extract in terms of MTT reduction. With the THO cells, the first extract reduced formazan production to 71.92+/-3.22% of that measured in control cells. This result was statistically different from controls. With the FFC cells, although both first extract and second extract cause a decrease in formazan production. to 79.8+/-14.7% and 79.3+/-16.18% respectively, these were not a statistically different from controls. The positive control extracts caused a marked, significant decrease in formazan production to 21.3+/-1.33% of the control value in THO cells and 7.73+/-1.71% in FFC cells respectively. The second extract did not cause a significant alteration in formazan production levels. This implies again that the toxic factor involved in the decrease of MTT reduction by HA-Spinel is removed by the first extraction in medium for 48 hours.

4.3.5. Expression of collagen synthesis

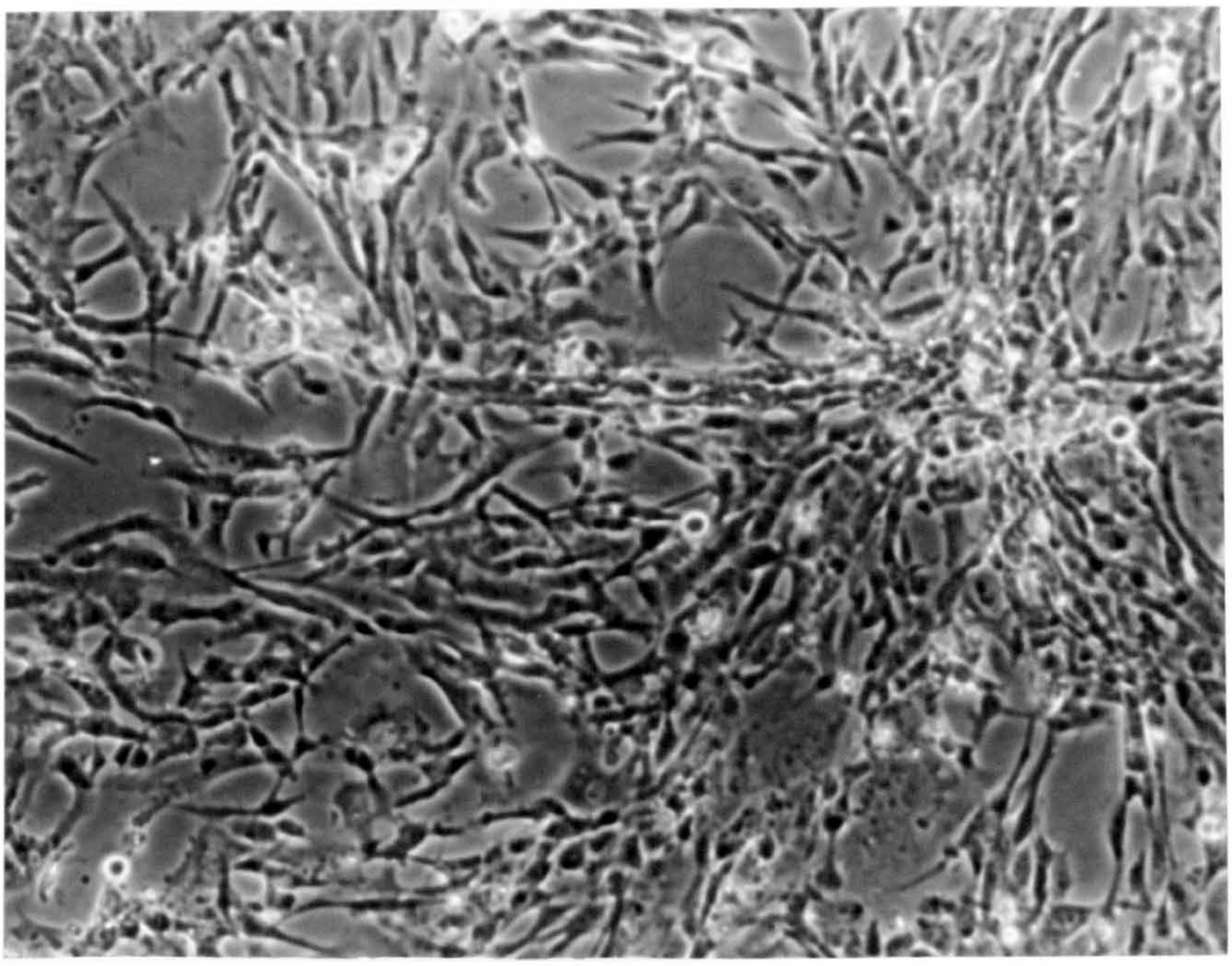
Figure **4.6** shows the collagen synthesis of THO (A) and FFC (B) cells cultured in the extracts of HA-Spinel and extracts of HA. In terms of the amount of collagen synthesis, the measurements were described as dpm (dpm is proportional to the amount of collagen synthesis).Similar to the results of GSH measurement, FFC cells appeared to be more susceptible to inhibition of the collagen synthesis from the HA-Spinel and the positive control extracts. With the THO cells, an effect of the first and second HA extracts on collagen synthesis was not apparent; collagen synthesis in the first extract was 90.8+/-12.66% of that measured in control and in the second extract was 105.9+/-12.80% of that measured in control. These results were not statistically different from control. The effect of the first HA-Spinel extract on collagen synthesis was apparent and collagen synthesis in first extract was 61.4+/-12.17% of that measured in control. This was statistically different from control. In contrast to the effect of the first extract, the second HA-Spinel extract did not cause a significant effect on collagen synthesis. With the FFC cells, as with the THO cells, the effect of the first and second HA extract were

not significant different from controls; collagen synthesis in the first extract was 96.2+/-21.43% of that measured in control and in the second extract was 106.9+/-14.4% of that in control. In contrast, HA-Spinel extracts cause a marked effect on collagen synthesis. After exposure for 48 hours, the first extract decreased collagen synthesis to 59.8+/-5.12% of controls, and the second extract decreased collagen synthesis to 77.6+/-3.96%. The results were statistically different from controls. The positive control extract caused a marked, significant decline in collagen synthesis level to 16.0+/-1.81% that of control values in THO cultures and 1.05+/-0.12% of control values in FFC cultures. To facilitate comparison of the collagen synthesis of THO and FFC cells in the extracts the data have been expressed as a percentage of the control values (Figure **4.7**). It shows that the extracts have a greater effect on collagen synthesis in the FFC cells, and emphasises the greater sensitivity of the FFC cells to the extracts, which were not as toxic as the positive control extracts. The percentages of total protein synthesis which is measured by ^{3}H-proline incorporation collagen protein in the THO and FFC cells cultured in the extracts are shown in Table **4.1**. This is not significantly altered by culture in any of the extracts in either cell type.

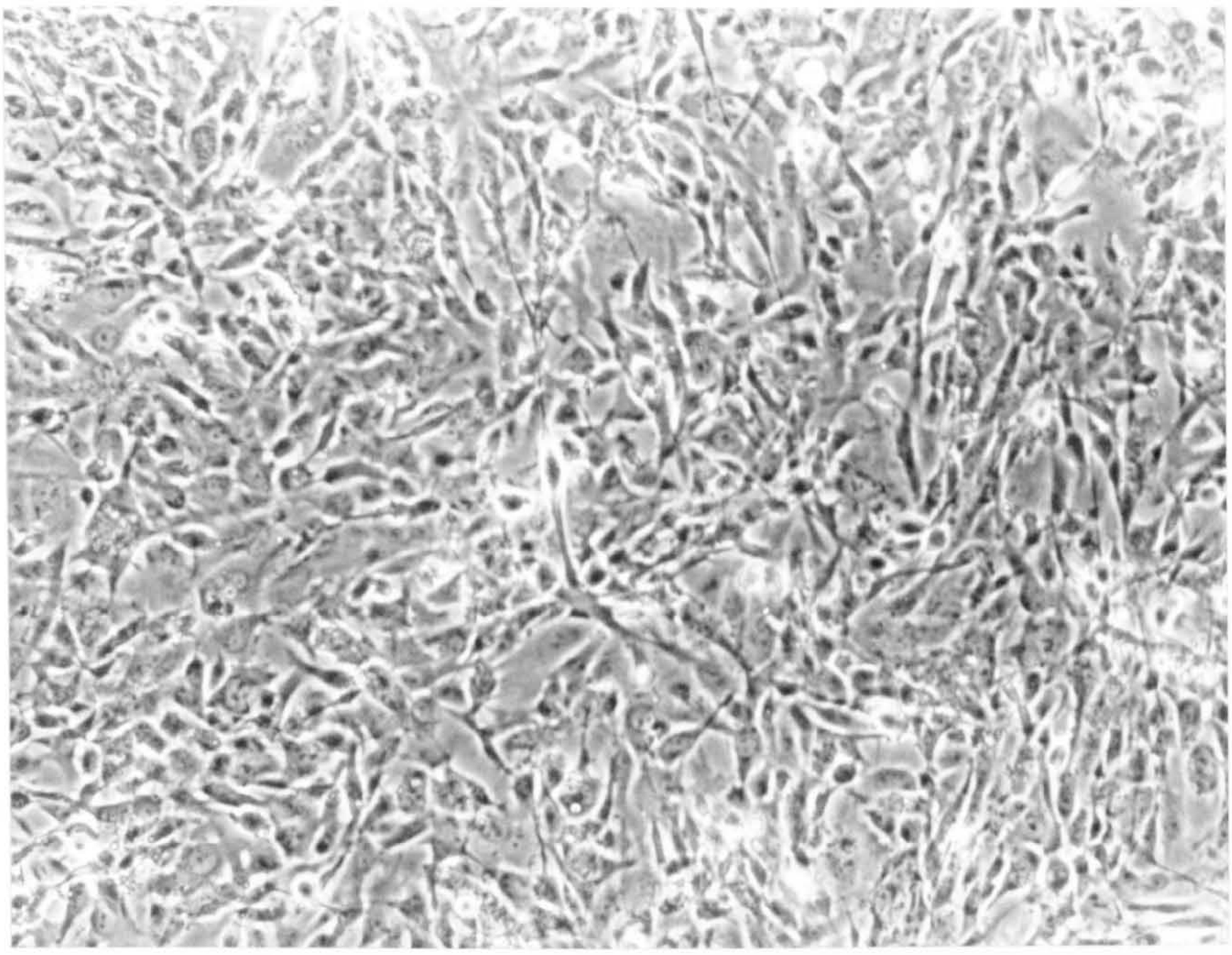
Table 4.1 Percentage of collagen protein in the total protein synthesised by THO and FFC cells in the extracts. Results are mean +/- SD, N=4. *P<0.05 by ANOVA followed by Dunnett's test, compared with control. P.C: positive control, N.C. negative control. Where HA48: the first HA extract; HA 96: the second HA extract; Be-48: the first HA-Spinel extract; AE-48: the second HA-Spinel extract; P.C: positive control.

Extracts	**% Collagen +/- SD** (THO cells)	**% Collagen +/- SD** (FFC cells)
Polystyrene	91.56 +/- 3.75	89.37 +/- 0.47
HA48	90.32 +/- 5.38	90.55 +/- 3.92
HA96	88.38 +/- 7.33	91.28 +/- 7.56
BE-48	92.08 +/- 4.85	84.75 +/- 2.86*
AE-48	91.84 +/- 4.52	83.86 +/- 5.39
P.C	84.38 +/- 10.49	99.74 +/- 13.95

(A)

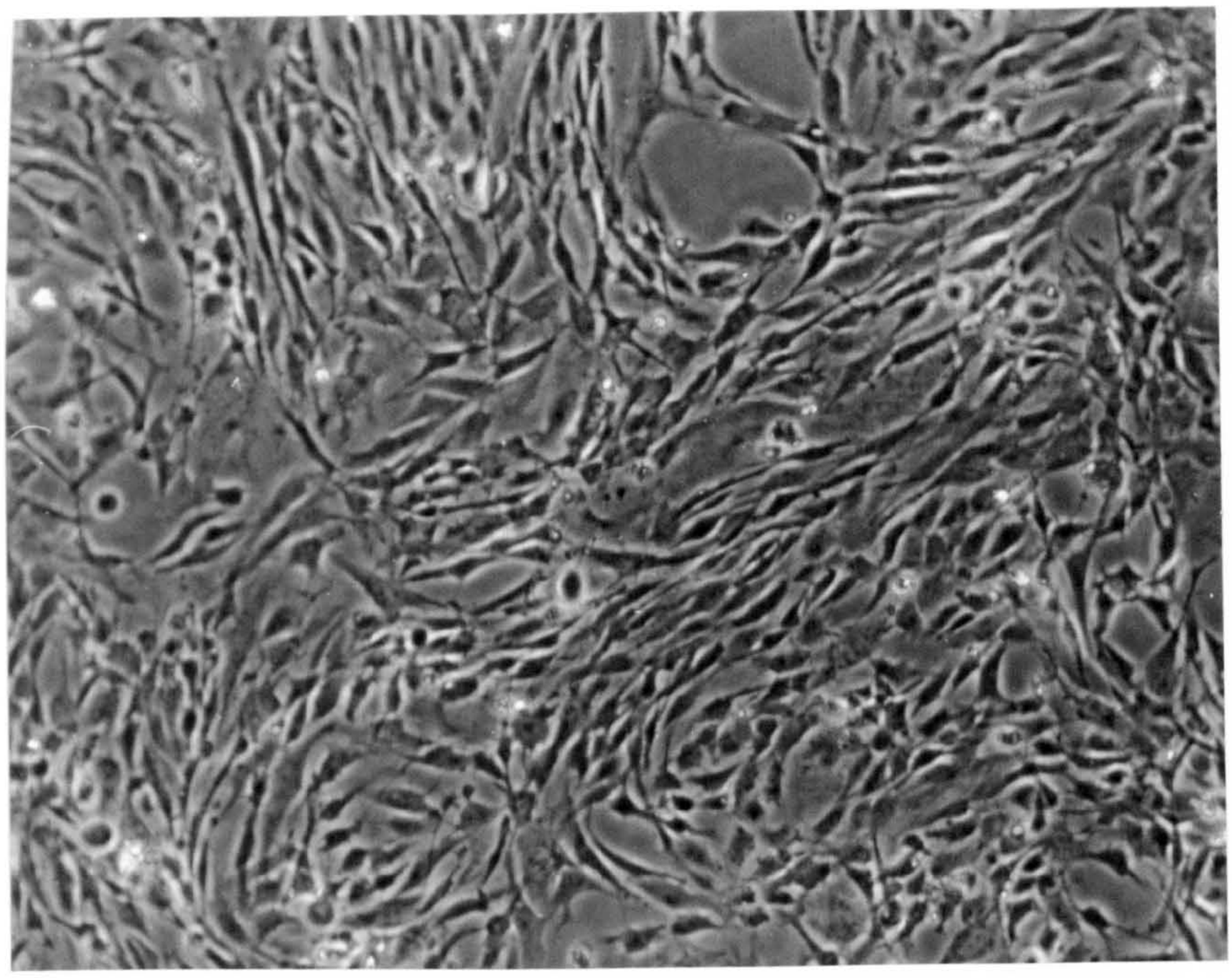


(B)



Photograph 4.1. Morphological observation of immortalised human osteoblasts (THO) and immortalised rat osteoblasts (FFC) in extracts; A, THO growth in control condition (Polystyrene); B, FFC growth in control condition (Polystyrene); Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells, and were incubated for 96 hours. Magnification: x440

(A)



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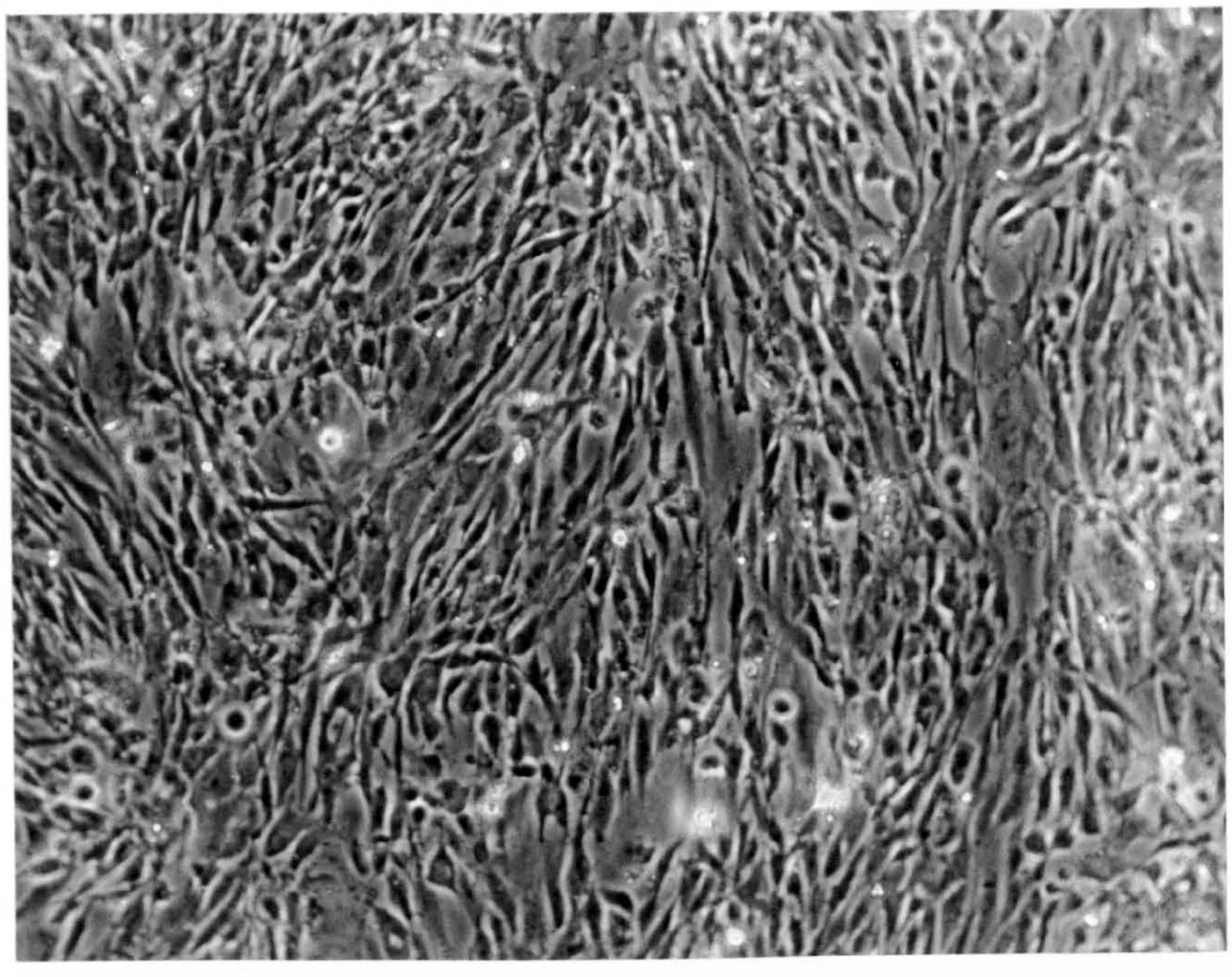
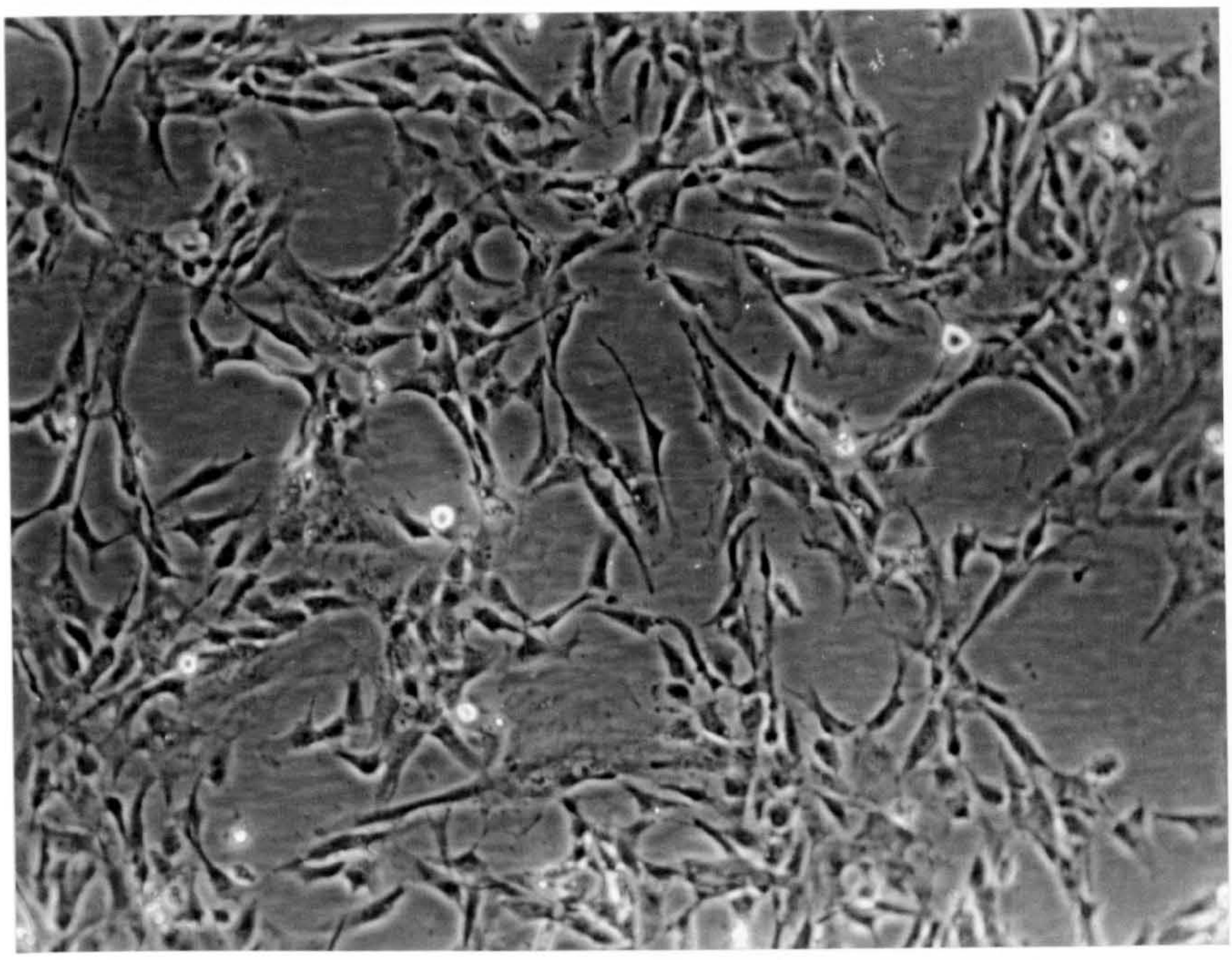


Photograph 4.2. Morphological observation of immortalised human osteoblasts (THO) and immortalised rat osteoblasts (FFC) in the extract; A, THO growth in first HA extract; B, FFC growth in first HA extract; Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells, and were incubated for 96 hours and expressing normal morphology. Magnification: x440

(A)



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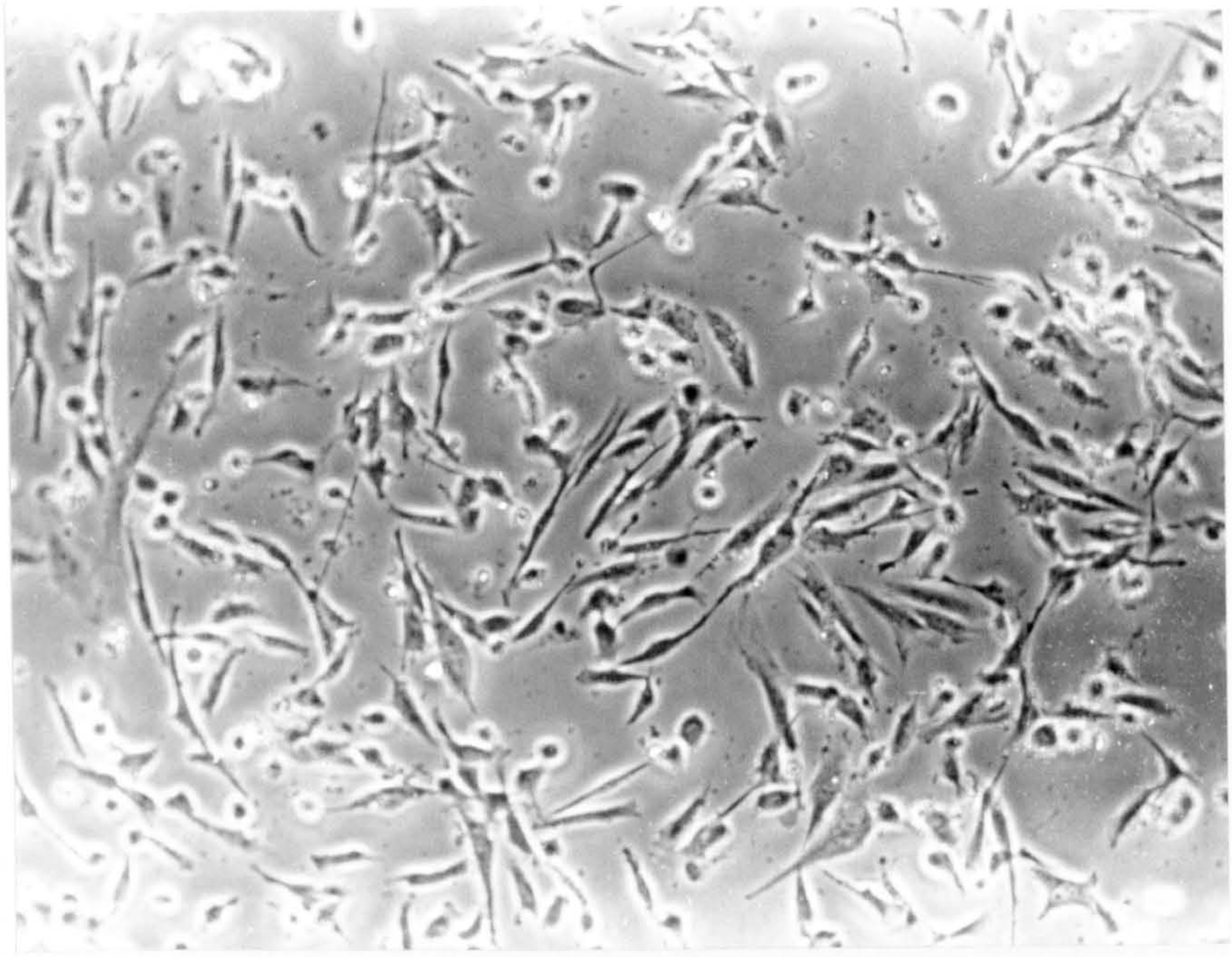
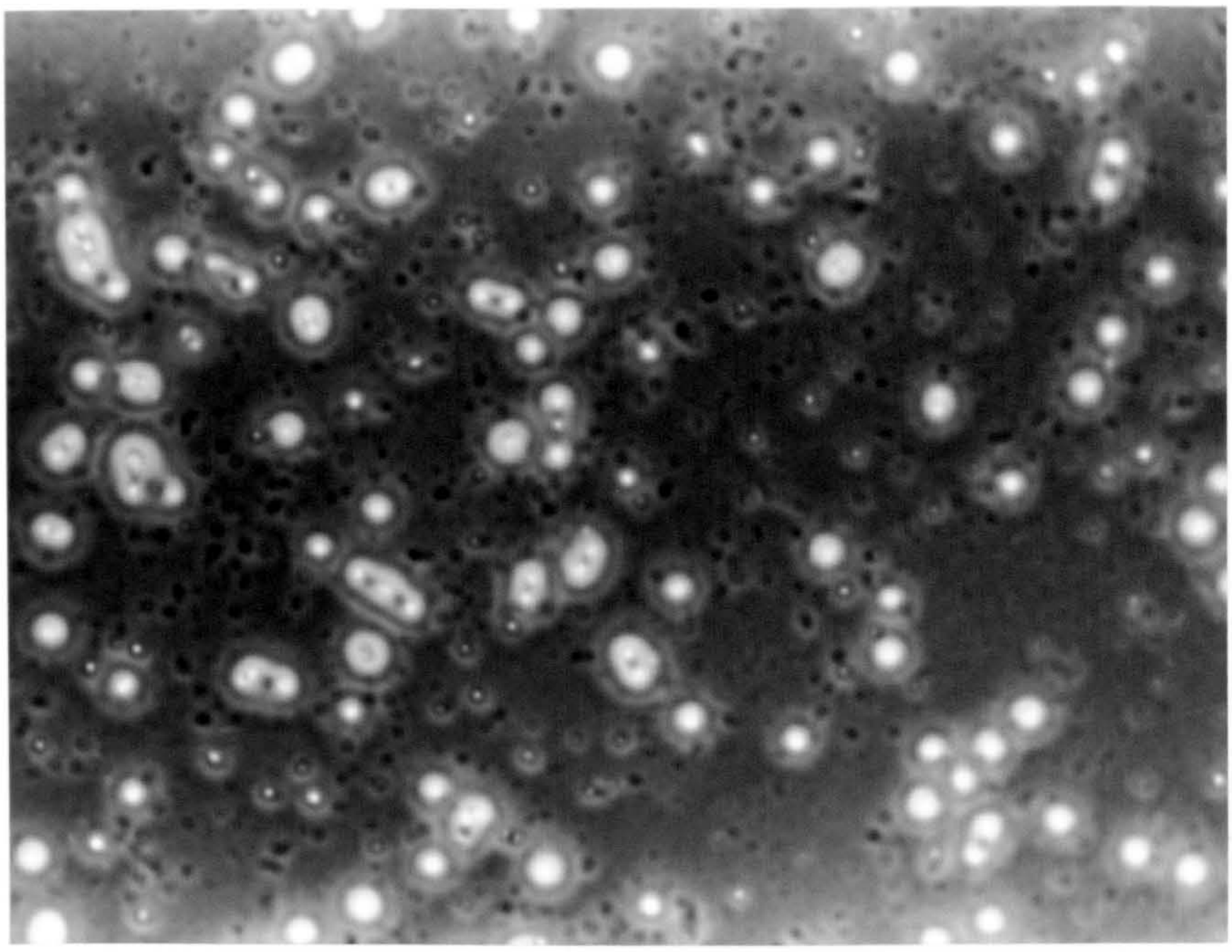
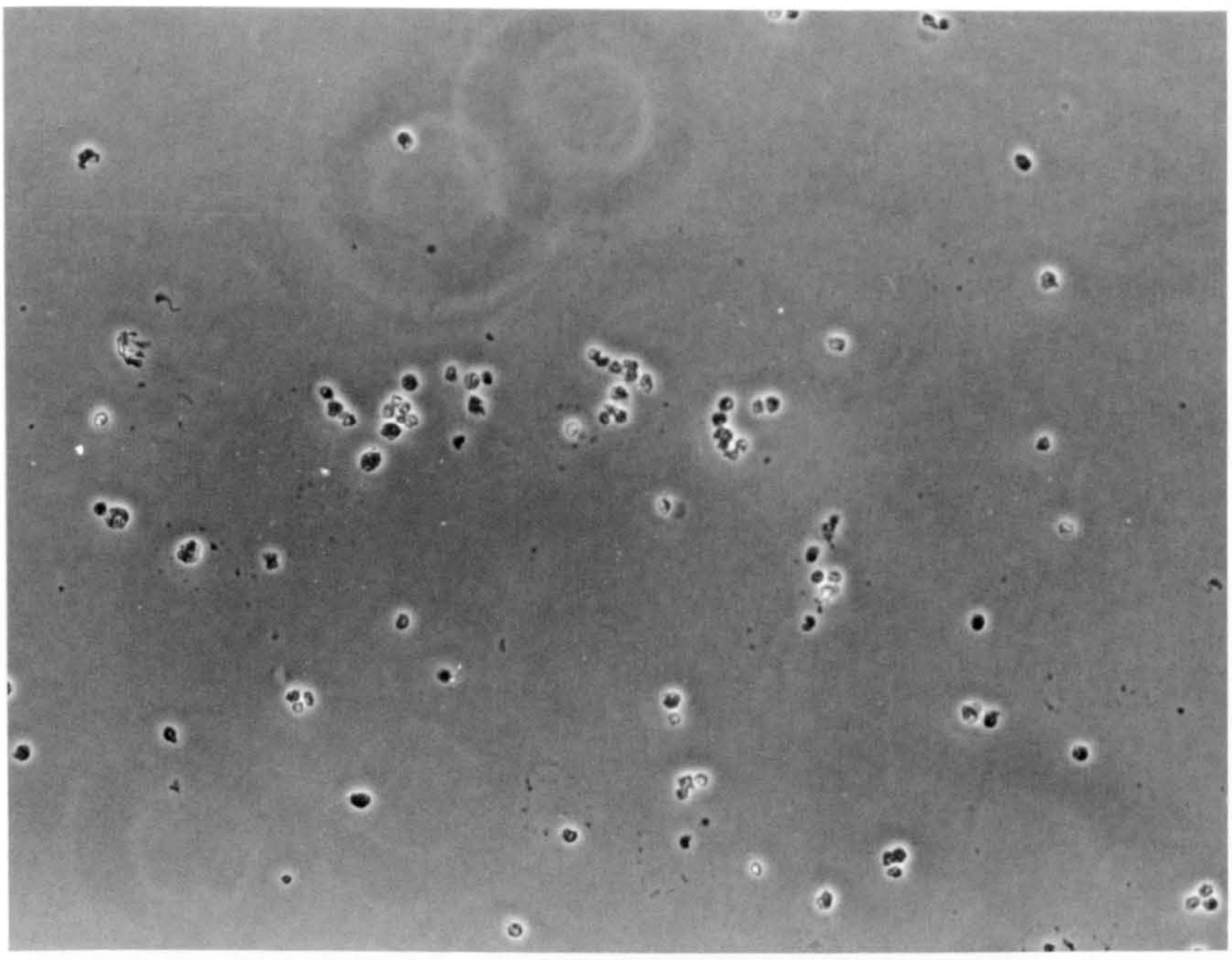


Photograph 4.3. Morphological observation of immortalised human osteoblasts (THO) and immortalised rat osteoblasts (FFC) in the extract; A, THO growth in first HA-Spinel extract; B, FFC growth in first HA-Spinel extract; Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells, and were incubated for 96 hours. Cell growth and proliferation were affected by the first HA-Spinel extract. Magnification: x440

(A)

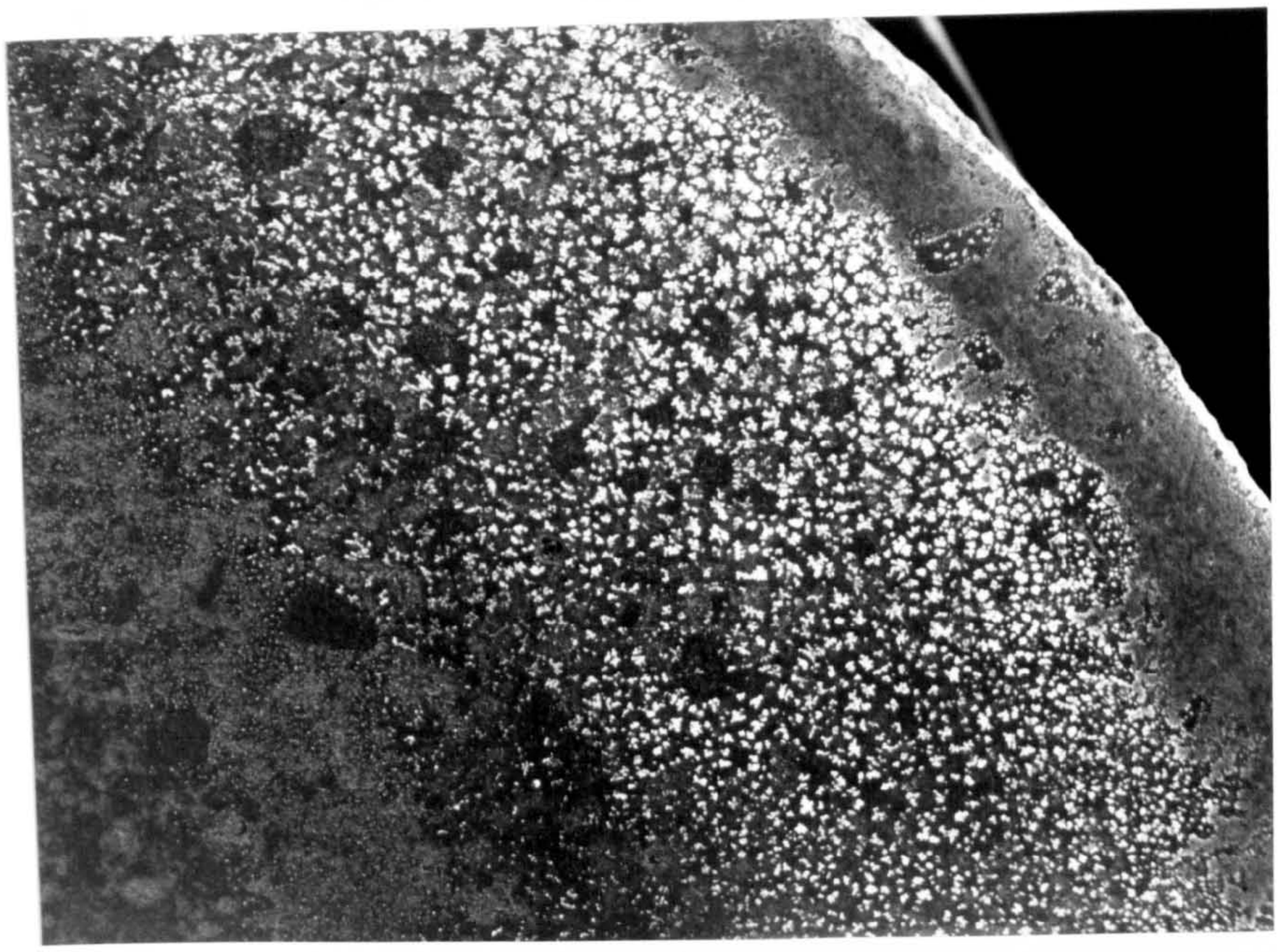


(B)

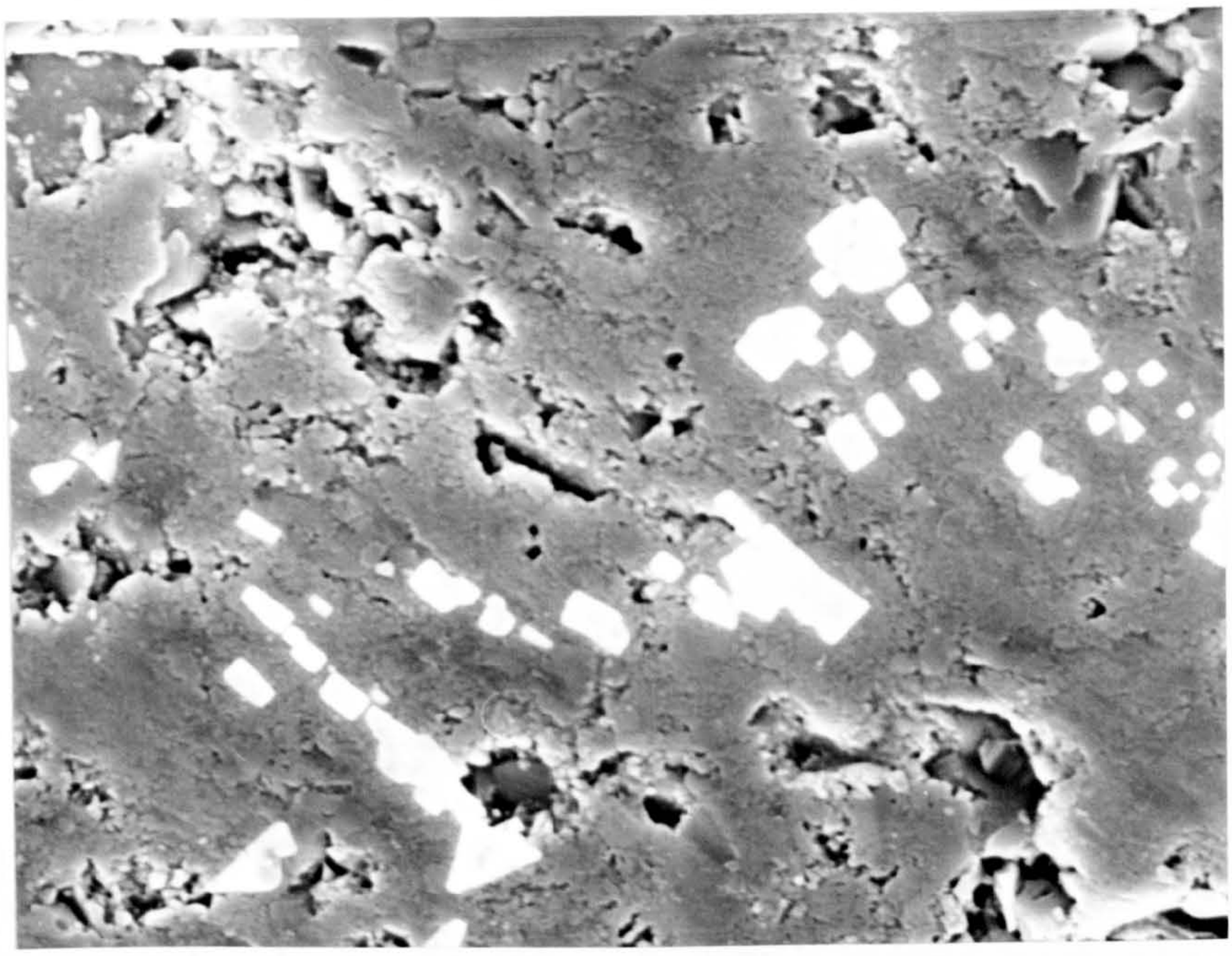


Photograph 4.4. Morphological observation of immortalised human osteoblasts and immortalised rat osteoblasts in the positive extract; A, THO growth in positive control; and B, FFC growth in positive control. Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells, and were incubated for 96 hours. Cells were damaged by toxic chemicals in the extracts. Magnification: x440

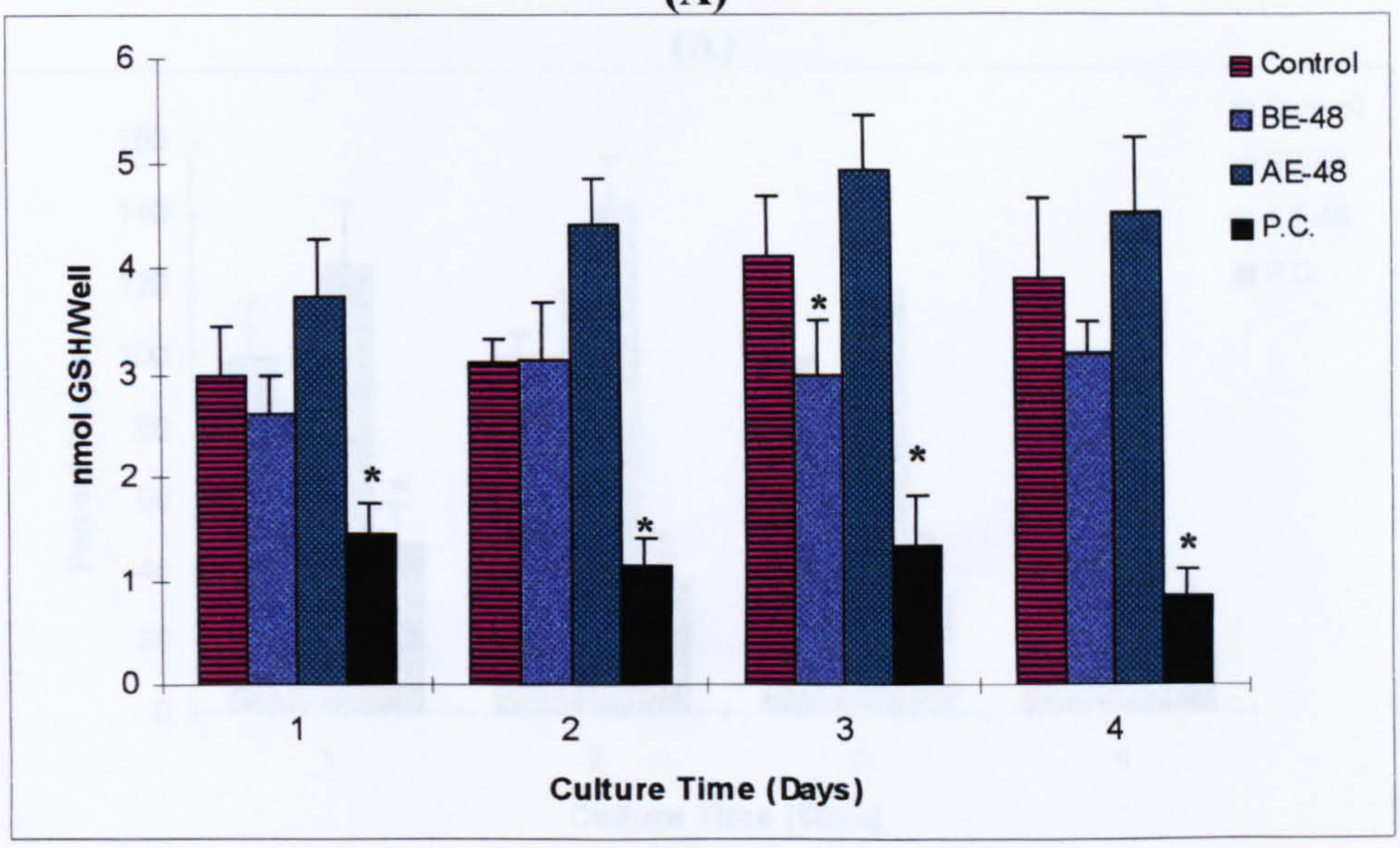
(A)



(B)



Photograph 4.5. Observation of the surface of HA-Spinel biomaterial by SEM, after 48 hr immersion in the culture medium (DMEM). The white particles are unidentified organic material. Magnification: x50 (A) and x3500 (B).



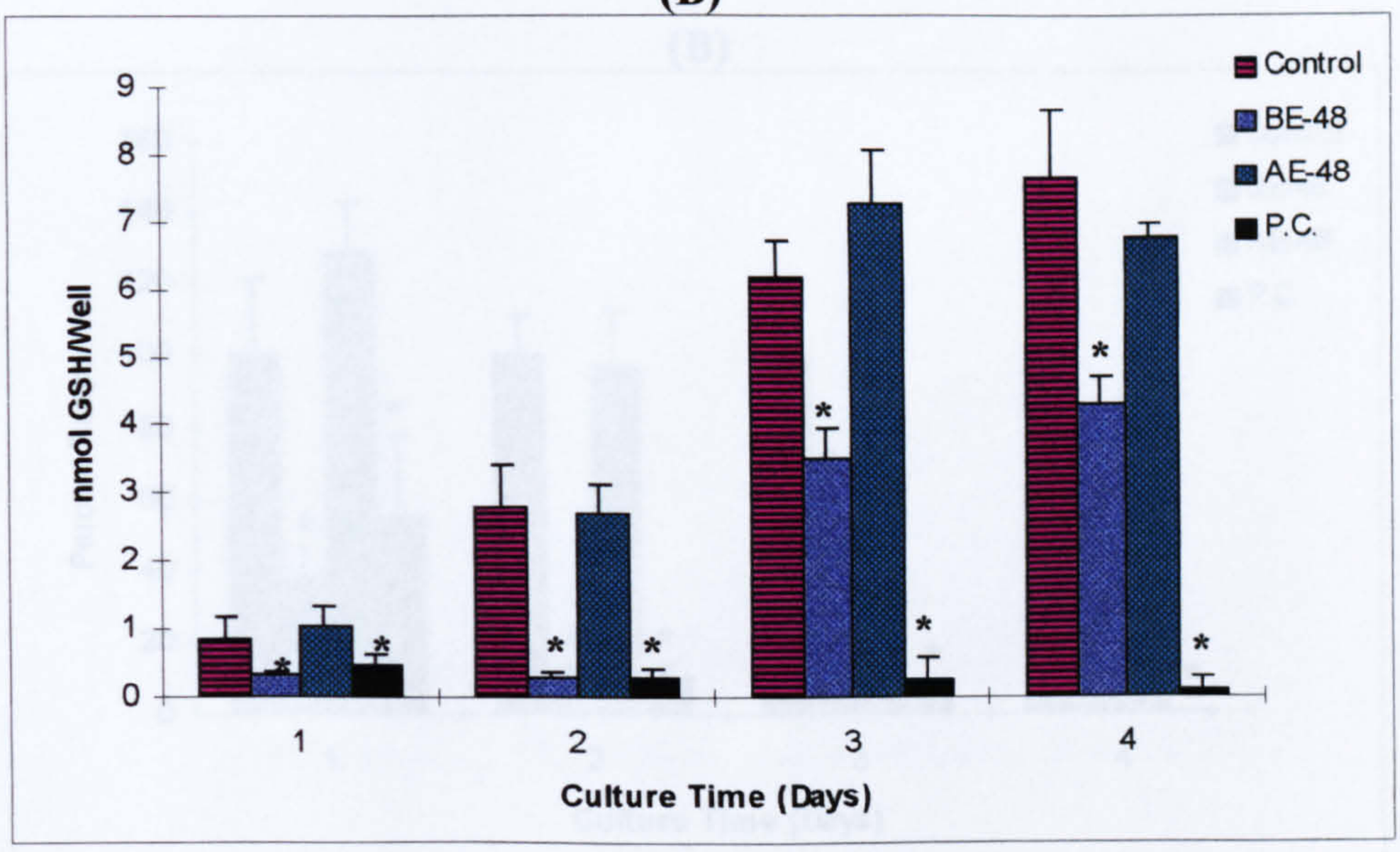
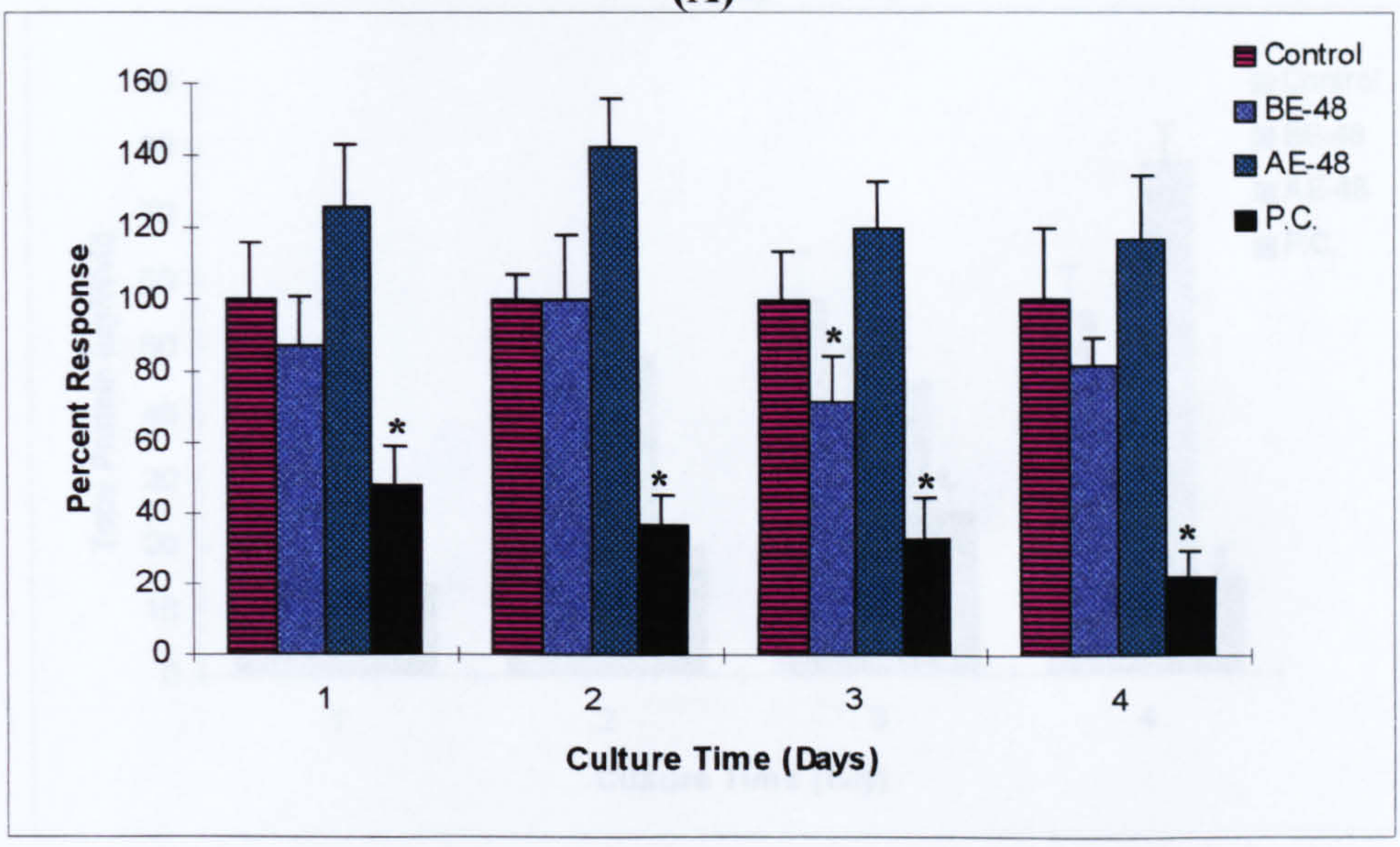


Figure 4.1.The responses of immortalised human osteoblasts (THO) (A) and immortalised rat osteoblasts (FFC) (B) to the extracts detected by GSH determination. Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells. The intracellular GSH was extracted with 200 µl aliquots of 10% trichloroacetic acid (TCA). Fluorimetric analysis of GSH content in the extracts was measured on a spectrofluorimeter (Shimadazu RF-5001 PC). BE-48, in first HA-Spinel extract: AE-48, in second HA-Spinel extract; P.C., positive control; Results are mean +/- SD, N=4, * $p<0.05$ by ANOVA followed by Dunnett's test.

(A)



(B)

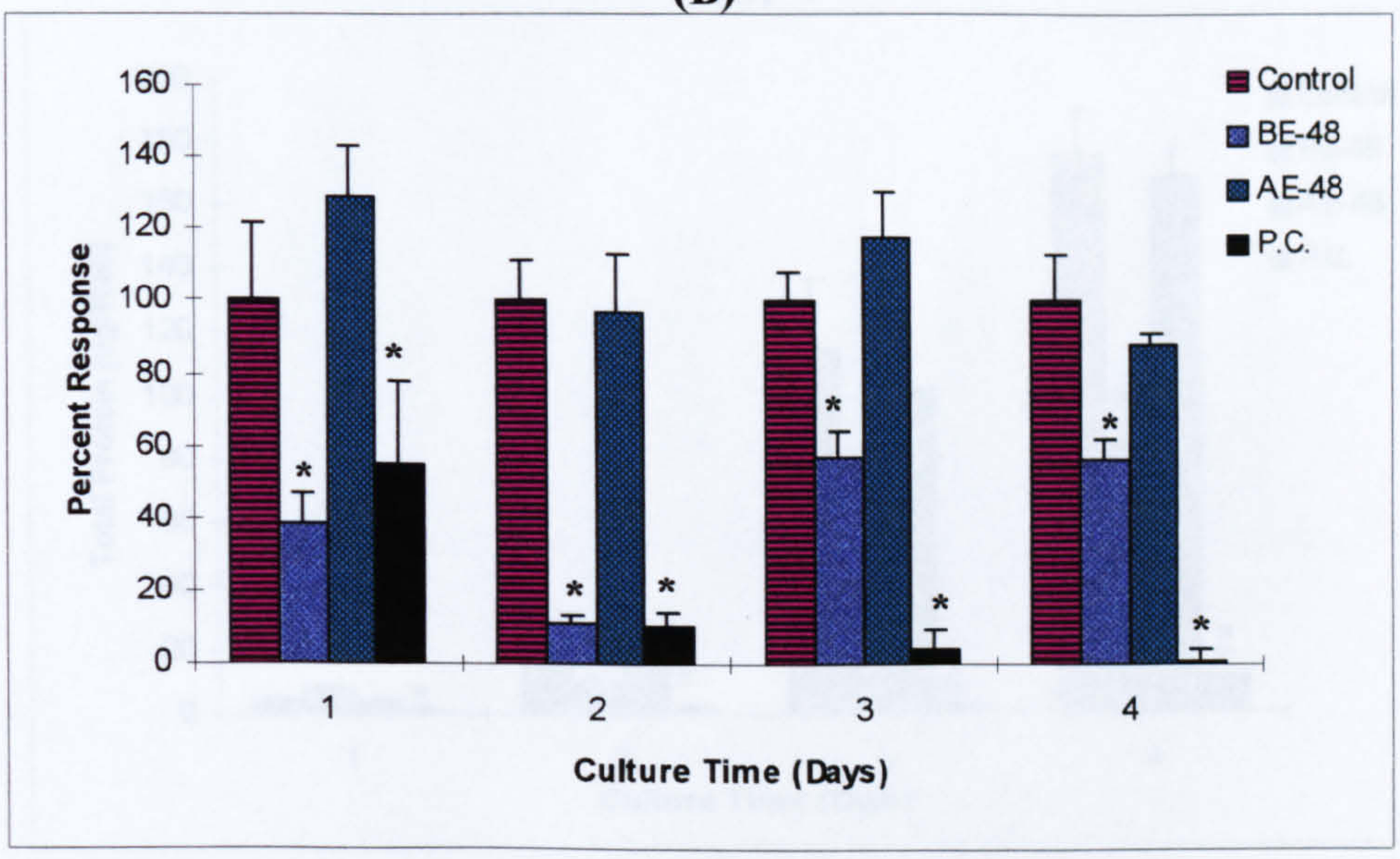
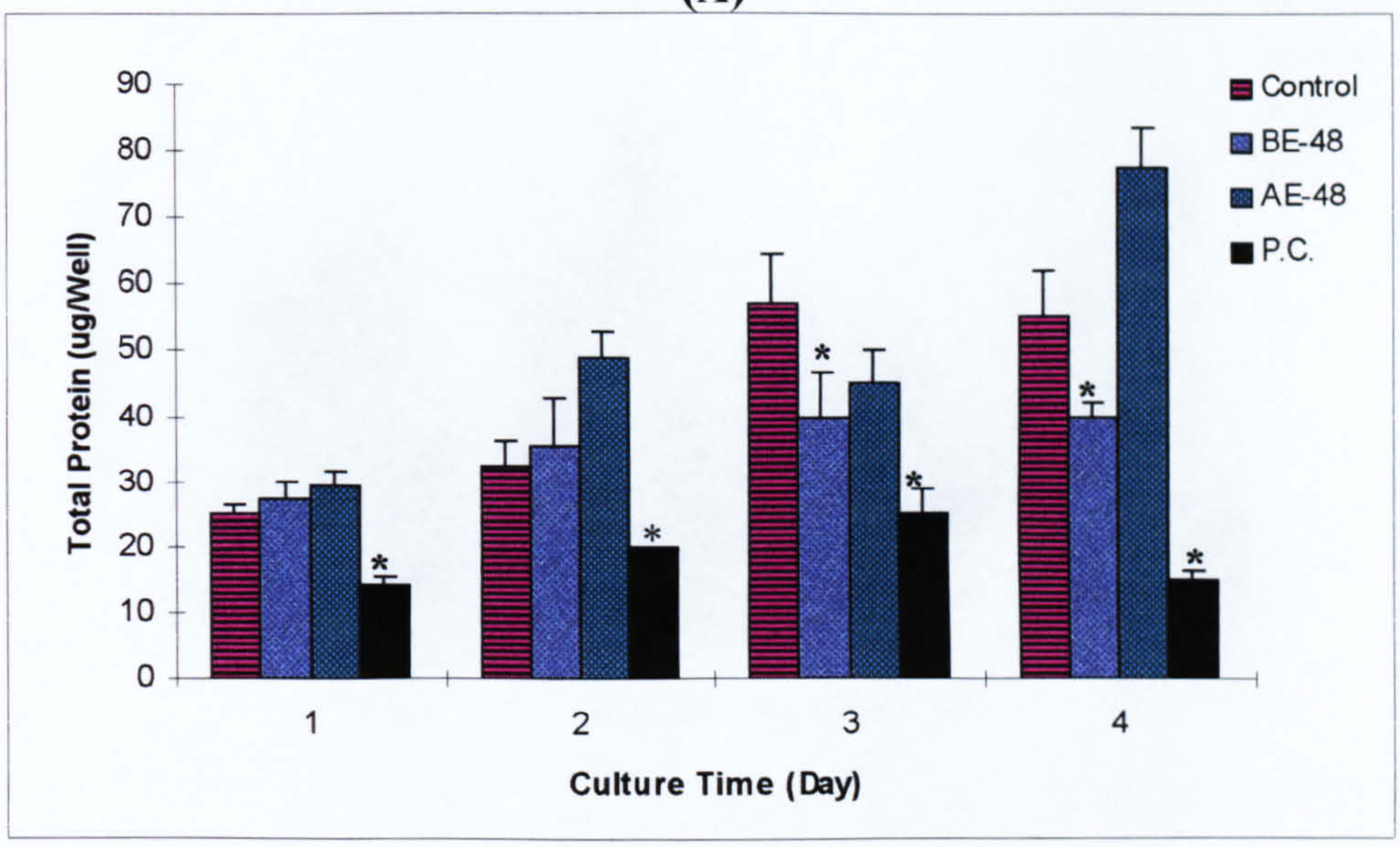


Figure 4.2. Percentage response of immortalised human osteoblasts (THO) (A) and immortalised rat osteoblasts (FFC) (B) to the extracts detected by GSH determination. Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells. The intracellular GSH was extracted with 200 µl aliquots of 10% trichloroacetic acid (TCA). Fluorimetric analysis of GSH content in the extracts was measured on a spectrofluorimeter (Shimadazu RF-5001 PC). BE-48, in first HA-Spinel extract: AE-48, in second HA-Spinel extract; P.C., positive control; Results are mean +/- SD, N=4, * $p<0.05$, by ANOVA followed by Dunnett's test. Expressing control data at all time points as 100%.

(A)



(B)

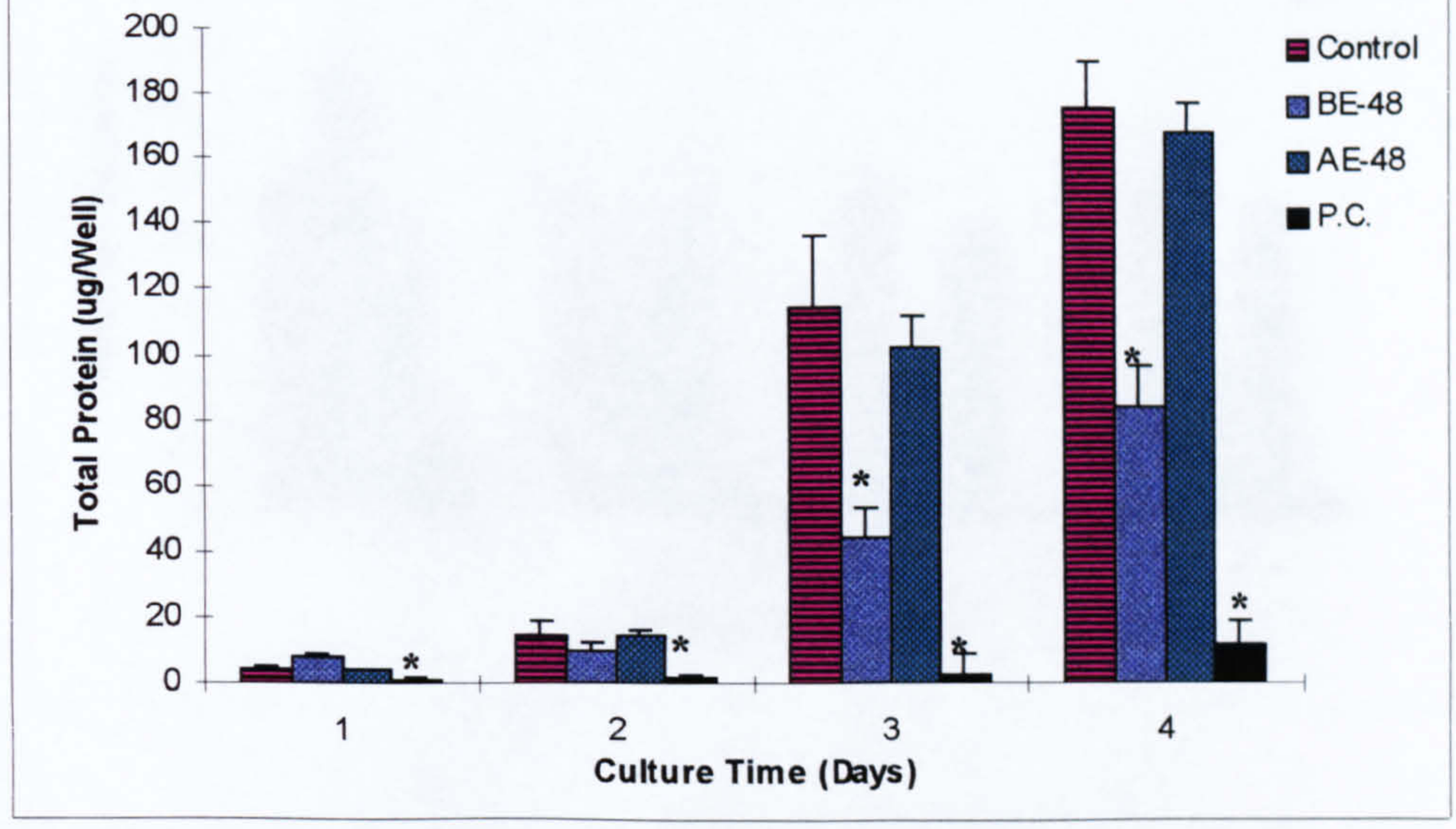
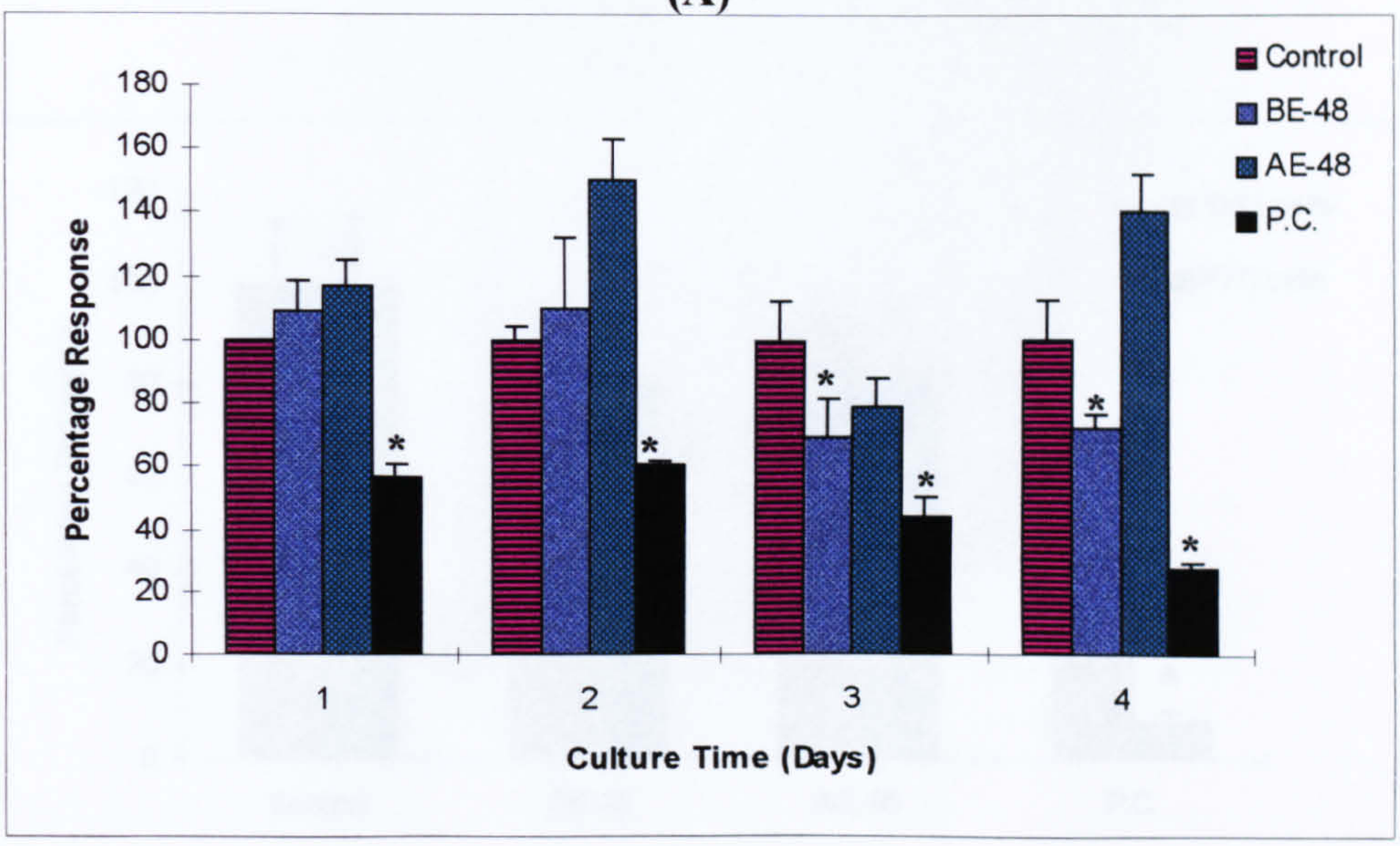


Figure 4.3. The growth (total protein) of immortalised human osteoblasts (THO) (A) and immortalised rat osteoblasts (FFC) (B) in extracts detected by Lowry Assay. Cultures were initiated in 24-well plates at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells. Cell protein was dissolved using 1 ml 0.5 M NaOH and total protein content measured on UV2101 PC Scanning Spectrophotometer. BE-48, in first HA-Spinel extract; AE-48 in second HA-Spinel extract, P.C., positive control; Results are mean +/- SD, n=4. * $P < 0.05$, by ANOVA followed by Dunnett's test.

(A)



(B)

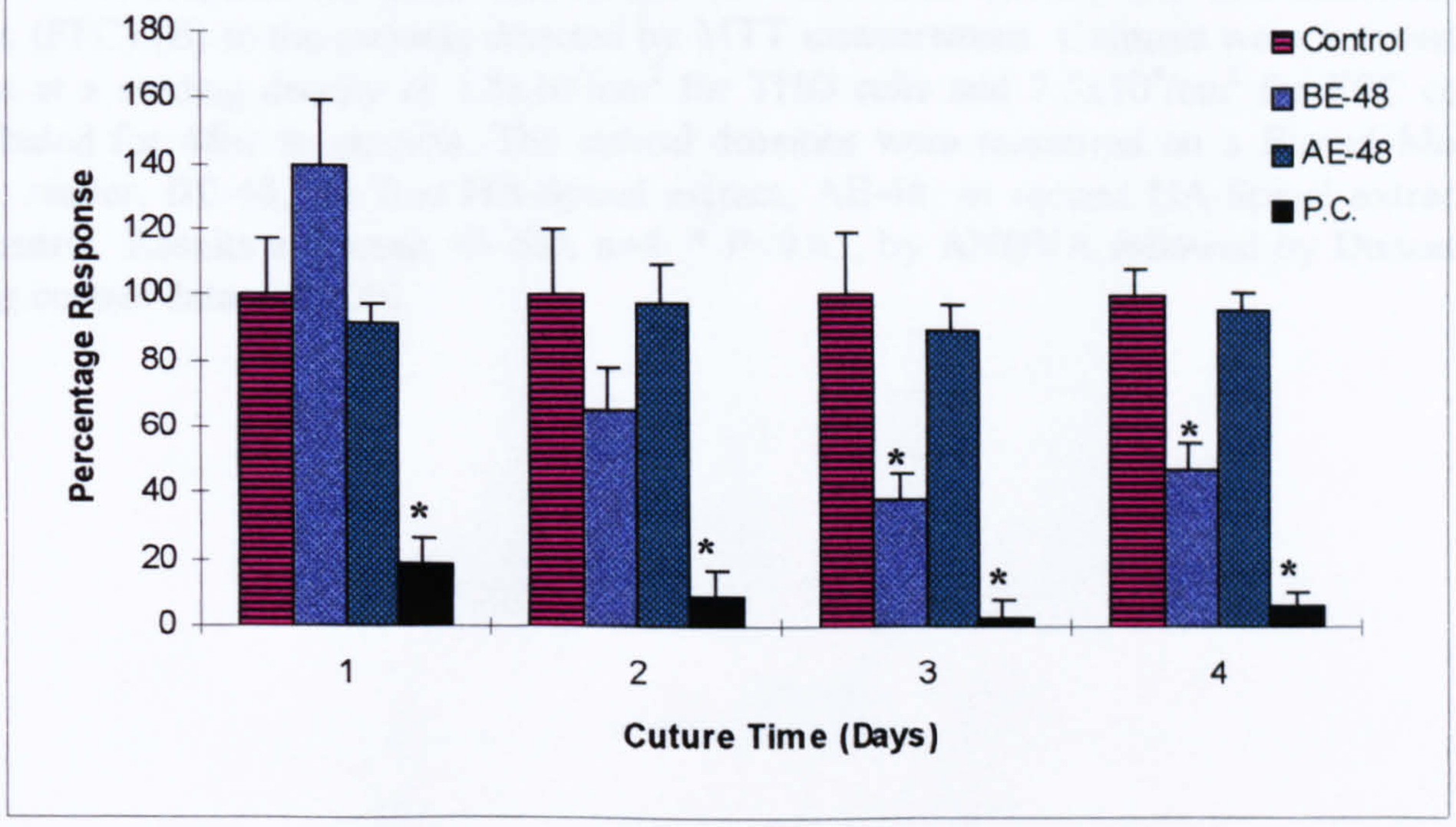


Figure 4.4. Percentage response of immortalised human osteoblasts (THO cells) (A) and immortalised rat osteoblasts (FFC cells) (B) to extracts detected by Lowry Assay. Cultures were initiated in 24-well plates ($2cm^2$ per well) at a seeding density of $5x10^4/cm^2$ for THO cells and $2.5x10^4/cm^2$ for FFC cells. Cell protein was dissolved using 1 ml 0.5 M NaOH and total protein content measured on UV2101 PC Scanning Spectrophotometer BE-48, in first HA-Spinel extract; AE-48 in second HA-Spinel extract, P.C., positive control; Results are mean +/- SD, n=4. * $P< 0.05$, by ANOVA followed by Dunnett's test. Expressing the negative control at all time points as 100%.

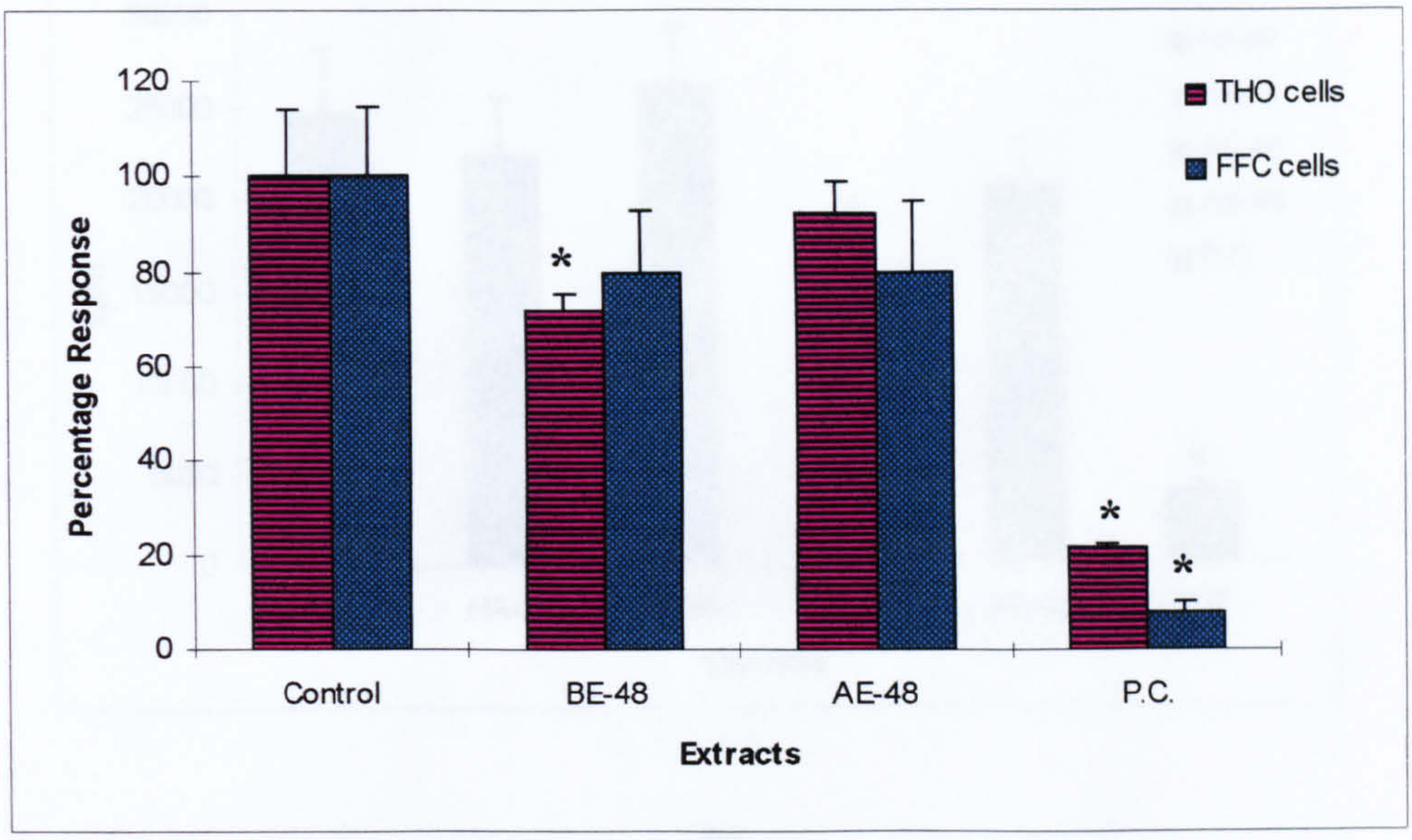
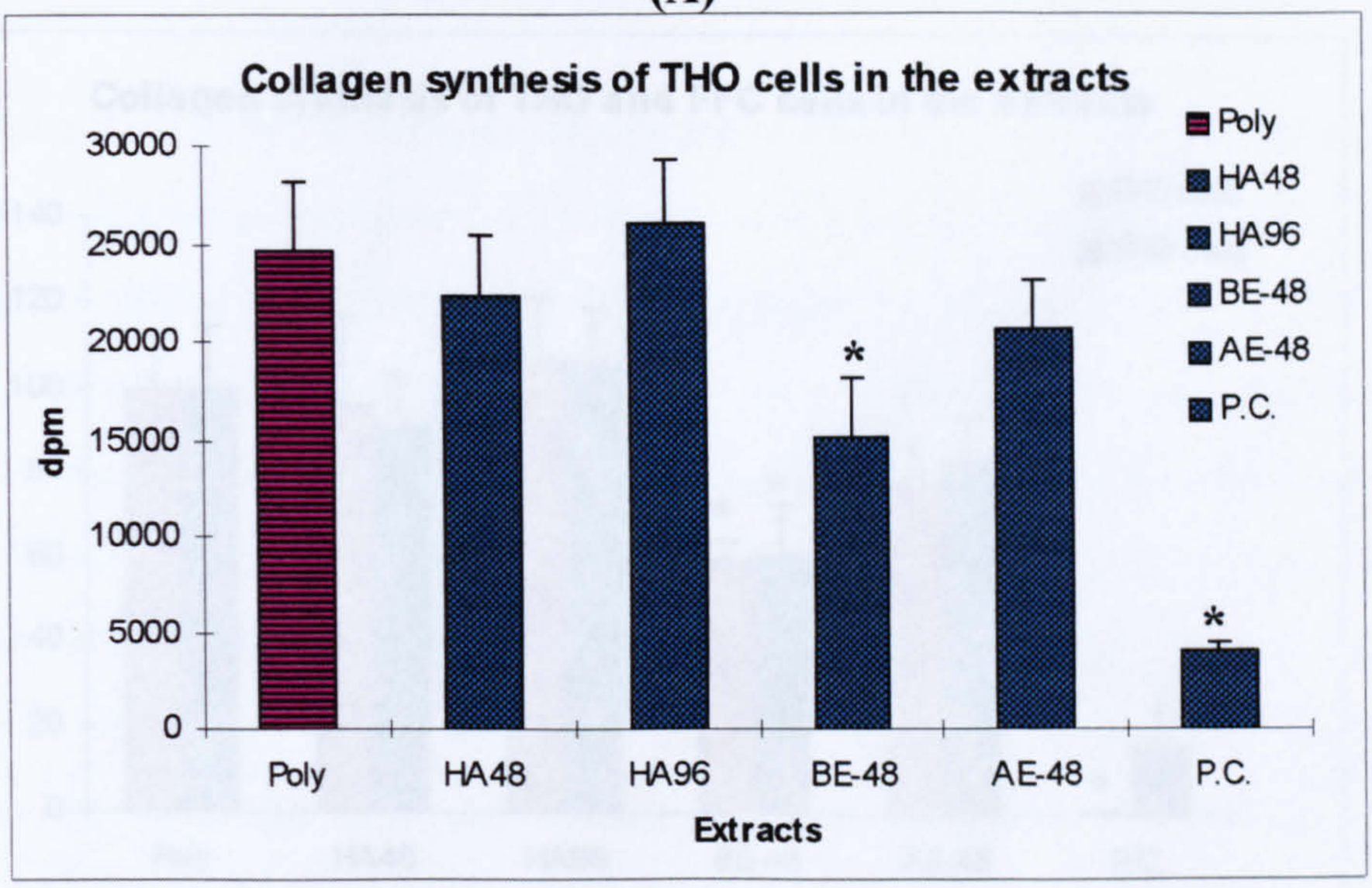


Figure 4.5. The response of immortalised human osteoblasts (THO) (A) and immortalised rat osteoblasts (FFC) (B) to the extracts detected by MTT measurement. Cultures were initiated in 96-well plates at a seeding density of $1.5x10^5/cm^2$ for THO cells and $7.5x10^4/cm^2$ for FFC cells, and were incubated for 48hr in extracts. The optical densities were measured on a Biorad Model 450 microplate reader. BE-48, in first HA-Spinel extract; AE-48, in second HA-Spinel extract; P.C., positive control. Results are mean +/- SD, n=4. * $P<0.05$, by ANOVA followed by Dunnett's test. Expressing control data as 100%.

(A)



(B)

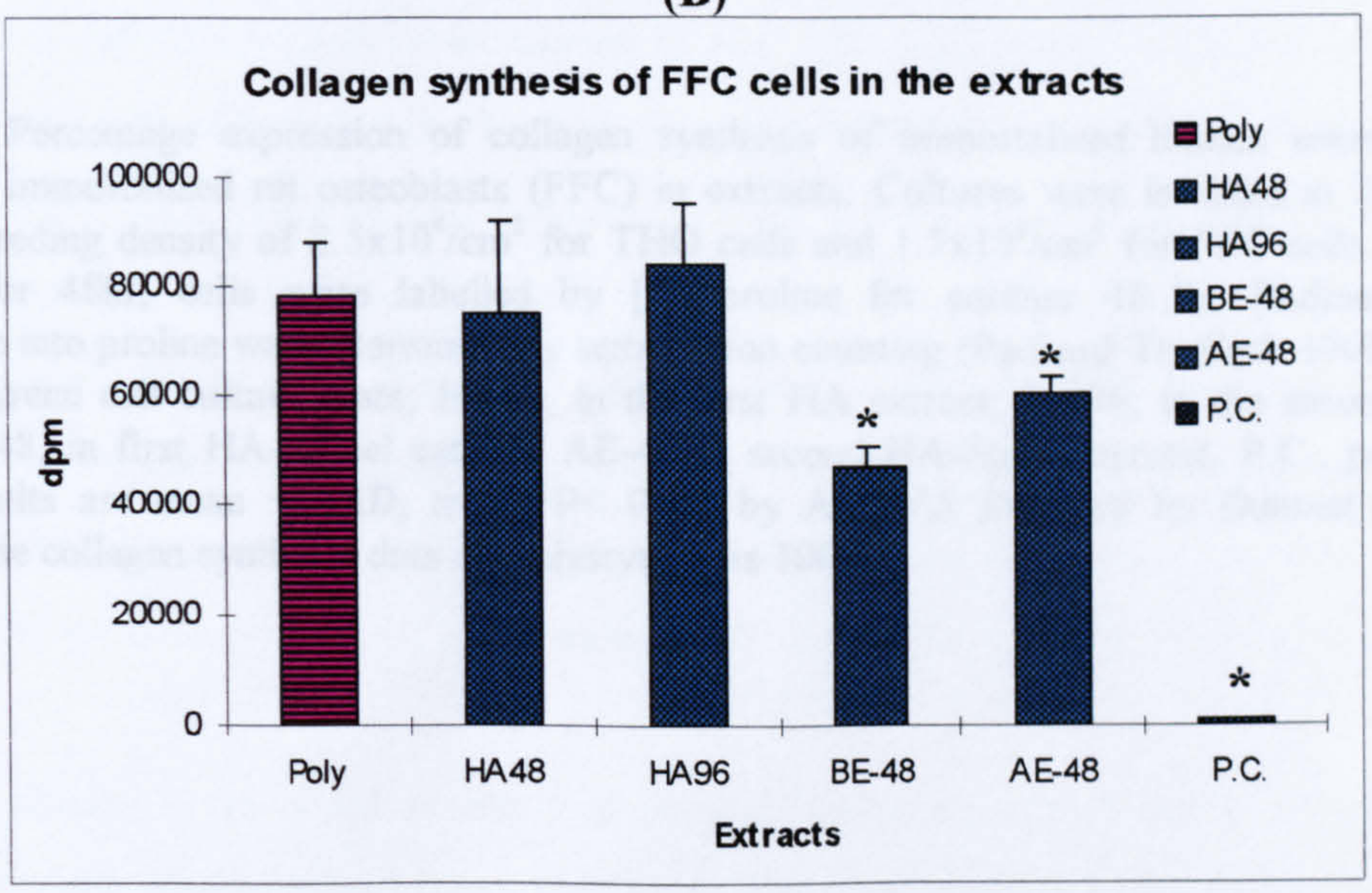


Figure 4.6. Collagen synthesis of immortalised human osteoblasts (A) and immortalised rat osteoblasts (B) in extracts. Cultures were initiated in standard 24-well plates at a seeding density of $2.5x10^4/cm^2$ for THO cells and $1.25x10^4/cm^2$ for FFC cells. After incubating for 48hr, cells ware labelled by [^{3}H]-proline for another 48 hr. Radioactivity incorporation into proline was determined by scintillation counting (Packard Tri-Carb 1900 TR). Poly: polystyrene cell culture plate; HA48, in the first HA extract; HA96, in the second HA extract; BE-48, in first HA-Spinel extract; AE-48 in second HA-Spinel extract, P.C., positive control; Results are mean +/- SD, n=4. * $P< 0.05$, by ANOVA followed by Dunnett's test.

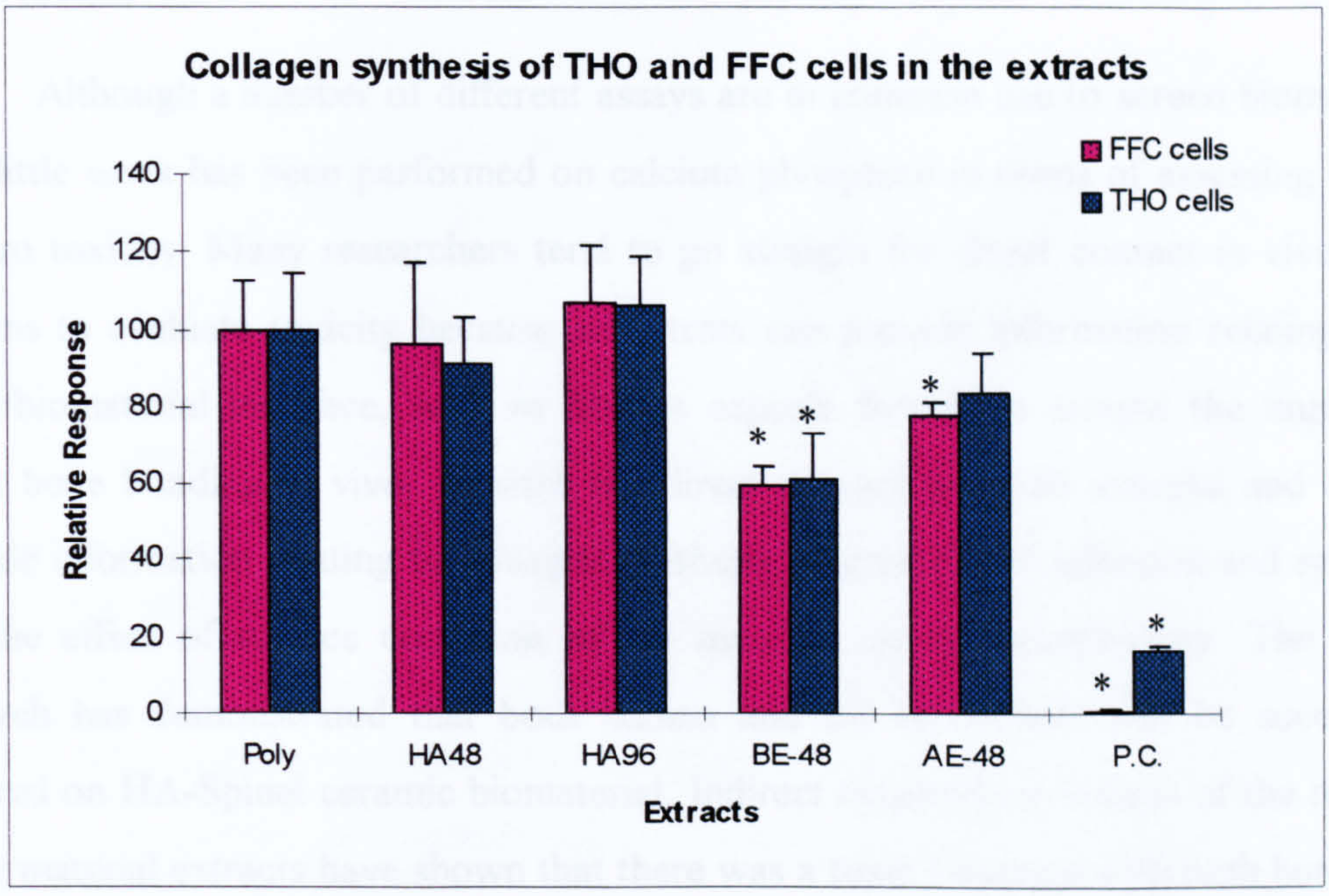


Figure 4.7. Percentage expression of collagen synthesis of immortalised human osteoblasts (THO) and immortalised rat osteoblasts (FFC) in extracts. Cultures were initiated in 24-well plates at a seeding density of $2.5x10^4/cm^2$ for THO cells and $1.5x10^4/cm^2$ for FFC cells. After incubating for 48hr, cells ware labelled by [^{3}H]-proline for another 48 hr. Radioactivity incorporation into proline was determined by scintillation counting (Packard Tri-Carb 1900 TR). Poly: polystyrene cell culture plate; HA48, in the first HA extract; HA96, in the second HA extract; BE-48, in first HA-Spinel extract; AE-48 in second HA-Spinel extract, P.C., positive control; Results are mean +/- SD, n=4. *P< 0.05, by ANOVA followed by Dunnett's test. Expressing the collagen synthesis data on polystyrene as 100%.

4.4 Discussion

Although a number of different assays are in common use to screen biomaterials, very little work has been performed on calcium phosphate in terms of assessing indirect in vitro toxicity. Many researchers tend to go straight for direct contact in vivo model systems to evaluate toxicity because such tests can provide information relating to the tissue/biomaterial interface, such as fibrous capsule formation around the implant, or direct bone bonding in vivo. In vitro the direct contact between material and cell can provide information relating to collagen synthesis, degree of cell adhesion and spreading and the effect of surface condition of the material on cell morphology. The present research has demonstrated that both human and rat osteoblasts can be successfully cultured on HA-Spinel ceramic biomaterial. Indirect cytotoxicity indices of the response to the material extracts have shown that there was a toxic response with both human and rat osteoblasts. Morphological observations of the cells after exposure to the extracts showed that the cells cultured in the second extracts expressed similar morphology as the negative control, with flattened cell bodies and long processes extending from the cell body. Although the morphology of cells in the first HA-Spinel extract appeared normal, the cell number, particularly the FFC cell number in first HA-Spinel extract, was decreased. This result demonstrated that the first HA-Spinel extract affected the cells, particularly FFC cell growth. It indicated that a slight toxicity was present in HA-Spinel materials. The positive control extract caused cells to detach and die, and was strongly toxic.

Glutathione is involved in many biological functions and is wildly distributed among living cells. The oxidised form, GSSG, is readily converted to its more common reduced form, GSH, by glutathione reductase. Depletion of GSH prior to loss of cell viability can be caused by several mechanisms. Electrophilic species released from materials may conjugate with GSH either spontaneously, or more commonly, enzymatically through glutathione -S- transferase. Redox reactions initiated by the material or leachable compounds from the materials may oxidise GSH to GSSG, which is then secreted from cells. In the present research, the results obtained by GSH determination demonstrated that compared with the negative control, the GSH content of cells cultured in the first extract of the materials was lower, indicating the depletion of GSH in cells grown in the first extracts. This could be due to conjugation of GSH with

chemicals leached by material or oxidation of GSH. It was interesting that for THO cells cultured in secondary HA-Spinel extract at all time points, and for FFC cells cultured in secondary HA-Spinel extract at time point day 1 and day 3, the GSH level was higher than control. The increase in GSH is thought to be an indication of cells protecting themselves against sub-lethal toxic insult (Rodgers et al, 1998). This can also be interpreted as an activation of cellular defenses to deal with sub-lethal concentrations of toxic chemicals in the extracts (Shi et al, 1998).

Total cell protein measurement by the Lowry assay is a sensitive toxicity index. Macnair and co-workers (1996, 1997) suggested that methodology of the protein assay ensured that only viable cells were measured as detached cells (non-viable cells) were removed prior to addition of NaOH to the samples to digest the protein. Results showed that the total protein concentration of cells cultured in the first material extracts was lower when the culture time was extended. For example, beginning from day 3, total protein synthesised both by THO and FFC in first extracts was significantly lower than that in control group. This was particularly the case with rat osteoblasts. Chemicals leaching from the material caused cytotoxicity, and as a consequence, the protein concentration in terms of the number of viable cells was lower than that of the negative control samples.

With both the measurement of GSH content and total cell protein, the FFC cells were more susceptible than THO cells, to both the HA-Spinel extracts and the effects of the positive control materials. We believe this is partly because FFC cells are faster growing cells, though the seeding density of the FFC cells was half of that of the THO cells. In Chapter 3, we have discussed the different growth rates. For example, when the seeding density is $4x10^4$ cells/cm^2, over 8 days was required for THO cells to grow to confluence in 25cm^2 flasks. If the conditions were the same, only 4 days was required for FFC cell growth to confluence. With FFC cells, if the seeding density was the half of the above density, 5 days was enough to arrive at confluence.

Results obtained by MTT analysis show cells to be metabolically active after treatment with the extracts, however, differences were observed with exposure to different extracts. MTT, a tetrazolium salt, is cleaved by intracellular dehydrogenases in all metabolically active cells, from its yellow soluble form into an insoluble, purple formazan product. This colorimetric method based upon the reduction of tetrazolium salts is aimed at measuring cell proliferation, and by this way to evaluate the toxicity of

the materials. This method relies on the concept that tetrazolium salts are readily reduced to their respective formazans by metabolically active cells, and it is commonly used for measuring cell growth, and estimating cellular viability in vitro. The main advantages conferred by MTT assay is that it can be performed on cells grown in microtitre plates using an automatic microplate reader. Compared with the results of the GSH determination and protein assay, for MTT, the difference between treatments of both human osteoblast and rat osteoblast and negative control were not so marked. This may imply that the MTT assay of cytotoxicity is not so sensitive as the GSH assay and Lowry assay. On the other hand, the biggest effects on the GSH assay and the total protein assay appeared from day 3. Accordingly, differences in the MTT assay may become apparent after day 3. Therefore, with the MTT assay, a longer time than 2 days in culture may have be needed to observe the effects of the extracts. There are other factors which affect the MTT assay, such as cell density, pH values and MTT concentration, etc. In the present research, the most important factor that affects the results may be the seeding density. The slight toxicity of HA-Spinel extract was not detected by MTT assay under the experimental conditions used.

The data in the present research have shown that HA-Spinel extract inhibits collagen synthesis of cells, particularly the FFC cells. This inhibition could come from Mg^{+2} and Al^{+3}, and/or even from small Spinel particles. These ions or particles may leach from the surface or crystal boundary of HA-Spinel materials during the first extraction. Another reason for inhibition may be that, after HA crystals were combined with Spinel crystals, the bioactivity of HA is limited, or decreased. HA material is considered to be bioactive and has an influence on collagen synthesis activities of bone cells. Gregoire et al (1990) demonstrated that HA particles can increase the synthetic activity of human bone cells (MC3T3-E_1) and their research demonstrated that there was a significant difference in collagen synthesis, as well as in ALP activity between cells cultured on HA particles and cells cultured on control material (culture plate). Thus, HA crystals in combination with Spinel crystals may sacrifice the bioactivity of calcium phosphate biomaterials, and will extend the time required for bone bond formation between implant material and hard tissue, and decrease the rate of osseointegration. Extracellular collagen synthesis is one of the most important function of osteoblasts. Type I collagen along with small amounts of types V and XII collagen make up approximately 90 percent of the organic matrix. The other 10 percent consists of non-collagen protein. In the present

research, the results show that even though the percentages of collagen synthesised in both THO and FFC cells were close, the amount formed varied markedly. Though the seeding density was only half of that of THO cells, the d.p.m. per well formed by FFC cells was approximately two fold over that of THO cells. The difference in radioactivity can be accounted for by the increased growth rate of FFC cells generating more cells which were in turn capable of more [^{3}H]-proline uptake. Meanwhile, the results show that although the first HA-Spinel extract did not affect the cell morphology, it indeed caused a decrease in collagen synthesis. However, it is difficult to ascertain whether the decrease in collagen synthesis was due to inhibition of cell growth or inhibition of collagen synthesis.

Extract of HA-Spinel biomaterial has no obvious effect on the morphology of THO or FFC when incubated the cells over a period 96 hours. It was not required to immerse the material in the culture medium before seeding cells and growing them on the material. This has demonstrated that even if the cells are able to attach and grow on the orthopaedic implant materials, this is not enough to identify whether or not toxic products would leach from the implants. There are many factors which could cause cell or tissue death in the human body. Toxicity elicited from orthopaedic material may induce an inflammation and exclusion reaction during early period of the clinical implantation. Because both the composition and structure of the surface are crucial to implant success, recently more attention has been paid to surface characteristics including surface biochemical characteristics and surface super-structure of the materials(Serre et al, 1993; Brian and Rena, 1994).

Considering the effect of an immersing solution on a material surface, Anselme et al (1996) found that it was impossible to culture osteoclasts on titanium with HA coating, if the material was not first immersed in culture medium for a couple of weeks. They implied that toxicity was present in the material. Takahiro et al (1997) described that the Zeta potential on material surfaces would be changed from -11 mV in a dry state to -26mV in culture medium when HA and bioactive glass ceramic were immersed in a physiological saline fluid. These observations also revealed that immersing solution has an important role in the modification of biomaterial surfaces. Katsufumi et al (1990) confirmed that the surface properties of bioactive glass ceramic (SS45:Si-Ca-P-O-H, F,) would be changed by altering the composition of the physiologic saline. Also Radin and Ducheyne (1994) declared that surface composition of materials was changed when the

composition of the immersing solution was changed. During immersion, Ca^{2+}, PO_4^{-3} in materials migrated into the immersing solution, and on other hand Ca^{2+}, Mg^{2+}, PO_4^{-3} in the solution was deposited onto the material surface, resulting in an altered composition and potential of the material surfaces. However, modification of biomaterial surfaces using immersing solutions is a complex process, and may be the proteins in the culture medium would contribute to the modification of the biological characteristics of the material surfaces.

After extraction, Ca, PO_4^{-3}, Al, Mg, tiny HA particles, and tiny Spinel particles may be present in the extract. Concentrations of Al, Mg, Ca could be determined in extract solutions by means of Atomic absorption spectrometry (Angerer and Schaller, 1999). This technique can be applied to almost all types of samples including alloys, polymers, ceramics, composites, and body fluids. However, it is limited to the determination of only one element at a time. A method called inductively coupled plasma-atomic emission spectrometry (ICP-AES) is used to determine trace elements in human fluids and could be applied to analyse the extracts. ICP-AES can analyse many elements, ether simultaneously or in a rapid sequential manner. However an important limitation of ICP-AES is that interference that may occur during analysis, and in the extracts this will come from medium components. A similar method named as inductively coupled plasma-mass spectrometry (ICP-MS) is also suggested to determine trace elements on the extract samples. ICP-MS has several prominent advantages over ICP-AES. For example, the detection limits are typically 10 to 1000 times better than ICP-AES, and within several minutes it can provide data on up to 75 elements, etc. Ca, Mg, HA particles, and Spinel particles and other organic and inorganic substances may be determined with ICP-MS, and ICP-AES techniques.

Normally, the concentrations of Al, Mg, Ca, and P in human serum are 0.131 μg/ml, 20μg/ml, 94μg/ml, and 43μg/ml respectively (Angerer and Schaller, 1999). It is difficult to judge the amounts of Al, Mg, Ca and P present in the extract as I have not measured those substances in the extracts. HA-Spinel materials are sintered together by tiny HA particles and Spinel particles. Mg_2O_4 has been well-crystallised during heat treatment at 800°C and during sintering at 1450°C and it is very stable in water (Mcgee,1974). However, a toxic reaction has indeed taken place in the first HA-Spinel extracts. When compared with cells cultured in HA extracts, collagen synthesis in HA-Spinel extracts was obviously limited. One possibility is that, compared with the HA-

Spinel material, more HA, as well as more Ca^{2+} and PO_4^{-3}, dissolved from the HA material. Researchers have reported that HA has stimulatory effects on cell growth and collagen synthesis (Gregorie et al, 1990), consequently, there were more cells, and more collagen was synthesised in the first HA extract. After making the first extracts, the material surface had been covered with protein, thus HA, as well as Ca^{2+} and PO_4^{-3}, dissolution was partially impaired by the protein layer. Then there was not so much HA, Ca^{2+} and PO_4^{-3} dissolved in the second extracts, so that differences in composition between HA extract and HA-Spinel extract has been decreased. Thereby the differences in cell growth and collagen synthesis in the second extracts were decreased. Another possibility is that during the first extraction, tiny particles of $MgAl_3O_4$ on the HA-Spinel materials surface entered the extract and were phagocyted by the cells. $MgAl_3O_4$ could not be enzymatically metabolised by cells, and its presence inside the cells inhibited cell growth and collagen synthesis.

Al is the third most prevalent element in the world naturally present in air, water, and soil. Between 10-100mg Al per day are ingested with food (Savory and Wills, 1991) and that of this approximately 1% is absorbed (Ganrot,1986). Orally ingested Al is excreted as Al phosphate. Absorbed Al is eliminated almost exclusively in the urine (<10 μg/day) in the case of people with normal health kidney. Prior to 1976, Al was thought to be non-toxic and of little significant to patient with chronic renal failure (Williams, 1981). Oral ingestion of Al containing compounds, phosphate binders are thought to be the source of Al in human serum. Highest Al concentrations are observed in bones, in spleen, and in liver. In addition, a tendency is found that Al concentration may occur in the brain of patients with chronic renal insufficiency who are treated with oral and/or Al-containing drugs. It is assumed that the permeability of blood-cerebrospinal barrier increases for Al in the patients and that this substance accumulates in their brain (Letzel et al, 1996). Normal brain grey matter Al content is less than 4μg/l. Grey matter Al content in patients has been reported 24μg/l (Williams, 1981).

Al administration has been shown to decrease bone mineralisation and inhibit osteoblast proliferation in the whole animals (Rodrigues et al, 1990). Al accumulates in erythrocytes and may enter osteoblast-like cells with the help of transferrin. Trace transferrin from the serum in the culture medium may mediate Al entering osteoblast (Kesai et al, 1991). In vitro and in vivo experiments support the possibility that Al may have a biphasic effect on bone growth. There have been previous reports on enhanced

collagen, protein, and DNA synthesis in osteoblast-like cells, and increased growth rate of 3T3 fibroblasts, caused by Al in vitro (Jeffrey, 1995). By use of immortalised rat osteoblast cell (FFC) culture to test Al cytotoxicity, McKay and co-workers (1995) found that when the concentration of Al in culture medium was lower than 500 μM, toxic reaction was not expressed on FFC cells. Furthermore, their results indicated that low concentrations of Al (0.5μM) may have a stimulation affect on the growth of osteoblasts. Lieberherr (1987) also reported that lower concentrations of Al (under 1.5 μM) increased osteoblast-like cell ALP activity, whereas higher concentrations (over 1.5 μM) were inhibitory. Lau (1991), working with chick primary calvarial cell culture and human osteoblast cell culture, showed that Al was mitogenic in these systems, increasing ^{3}H thymidine incorporation into DNA. Furthermore, correlating well with the in vivo studies of Quarles et al.(1989), they found the effects was regulated by hormones. Above 50μM, the effect was reversed and Al become inhibitory. Stanislawski et al (1999) also demonstrated the biphasic effect on bone growth by human dental pulp cell culture. They reported that when the concentration of Al in culture medium was lower that 1 mM, no cell death was found and the cell viability was same to control (glass culture slide). When the concentration was over 1 mM, the viability decreased rapidly. They suggested that Al^{3+} was present in quantities too small to account for a toxic effect on pulp cells. In the present research, I assume that the concentration of Al in the HA-Spinel extract was not too high since HA-Spinel ceramic is a bioinert ceramic and, at room temperature, it is impossible for Al^{3+} ions to migrate from the internal solid material into the extracts. Therefore, I do not think that the cytotoxic effects of HA-Spinel extracts are due entirely to Al ions, although they may have a contribution.

Up till now, very few reports exist related to the type of organic molecules deposited or grown on the surfaces of biomaterials. In the present investigation we found that organic material was deposited on the surface when HA-Spinel was immersed in culture medium. By detailed SEM observation the deposits have different morphologies. Part of the deposits are shaped in the form of regular crystals and some of the deposits shaped in the form of pins. Unfortunately, by SEM observation and energy disperse X-Ray analysis (EDXA), although we detected that the deposit was not calcium phosphate, we can not yet identify it. The result suggested that during immersing in culture medium, not only could the inorganic composition of biomaterial surfaces be altered, but also the

biochemical properties of the biomaterial surface would be modified due to the deposition of organic material from the culture medium onto the material surface. If we used the same specimen to make the second extract, there was no toxic response from either FFC cells or THO cells to the second extract of the HA-Spinel material (see Figure **4-2**, **4-4**, **4-7**). It was obvious that the biochemical properties of the material surfaces had been modified by immersing treatment. For dense orthopaedic materials made of metals or ceramics, only the material surfaces could be modified; the immersing media or extracting solutions are unable to penetrate into the interior of the dense materials at room temperature, to remove toxic components from the material interior. Supposing that the materials are degradable or corroded by body fluids, the toxic component under the material surface would leach out, and result in acute or chronic toxicity to the tissues. This will contribute to inflammation or rejection reactions, which are common clinical symptoms.

In conclusion, this approach demonstrated that a slight toxicity was present in the HA-Spinel biomaterial. It could be one of the reasons for production of acute inflammation during the early period of implantation. Immersion treatment has an important role to play in modifying the biochemical features of the biomaterial surface. By immersing the material in culture medium for 48 hr, the toxic response of both human and rat osteoblasts to the material was reduced. The results of this approach suggested that the HA-Spinel biomaterial should be a good candidate for orthopaedic implants.

Since the result revealed that a slight toxicity was present in this bioceramic material, the author suggests that acute inflammation during the early period of clinical implantation, is partially produced by chemicals leaching out of the implants. Immersing treatment of the biomaterials has an important role to modify the biochemical characteristics of the biomaterial surface. SEM observation found that during the immersion of the HA-Spinel biomaterial in the culture medium, some organic material was deposited on to the material surface. The results, meanwhile, demonstrated that after the biomaterial was immersed in culture medium for 48 hours, the toxic response of both THO and FFC cells to the biomaterial could be eliminated and both THO and FFC cells grow and proliferate on the material normally. The results suggest that HA-Spinel may be a good candidate for orthopaedic implant, and improved biocompatibility of the orthopaedic implants could be achieved by an immersing process.

Chapter 5 Collagen synthesis of osteoblasts grown on calcium phosphate biomaterials

5.1 Introduction

Collagen synthesis is the most important function of the osteoblast-like cells. Osteointegration between implant artificial organs and hard tissues is fulfilled by newly forming bone which is derived from the extracellular organic matrix on the implant materials. The successful synthesis of collagen protein on biomaterials has become a very important test for the biocompatibility of bone substitution materials both in vivo and in vitro. Extracelluar matrix has two important functions: it mediates the combination between tissues and substitute materials and it provides the substrate for mineralisation. Itakura et al (1998) suggested that cellular metabolism leading to mineralisation is the most sensitive indicator of osteocompatibility by which to test the biocompatibility of potential biomaterials. Ultra-structural study of the cell-material interface has also shown the formation of a thin layer of organic non-mineralised matrix and sparse patches of collagenous material in contact with titanium or titanium alloy (Bouvier et al 1994). Researchers have demonstrated that material composition and structure promotes the differentiation of osteoblasts and the deposition of a calcified cementlike boundary matrix, and this precedes the appearance of morphologically identifiable collagen fibres(Dubois et al, 1998; Sautier et al, 1994).

Collagen is a large and expanding family of proteins, which are defined by the presence, in at least a part of their sequence, of repeats of the triple helix Gly-X-Y, in which roughly one-third of the X repeats are proline and one third of the Y repeats are OH-proline. The collagen molecule is composed of three chains. In lamellar bone, type I collagen fibres are strictly parallel (van der Rest, 1991; Buckwalter and Cooper, 1987, 1995). Type I collagen comprises about 90% of the total extracellular matrix collagen and serves as an anchorage platform for cell attachment, and provides the infrastructure for mineral deposition. Zhong et al(1994) suggested that collagen is the connective tissue with negative surface potential which can induce the precipitation and crystallisation of calcium phosphate to its surface in a simulated body fluid (SBF). The negative surface potential increases the activities of cations, which result in precipitation and

crystallisation. De Bruijn et al (1995) suggested that osteoblasts first form mineralised globular accretions that subsequently fuse to form a substrate in which collagen fibres become grouped. Many research techniques are directed towards investigating the bone-bonding and biodegradable properties of calcium phosphate biomaterials in vivo. Little is known, however, of the cellular processes that underline bone formation on biomaterials, and bone bonding or biodegradation behaviour. Several important methods are in use to quantitatively and qualitatively measure collagen in vitro and in vivo.

Imaging observation by microscopy analysis:

By use of high energy electrons of extremely short wavelength and based on the advantages of high resolution and high magnification, electron microscopy (EM), scanning electron microscopy (SEM), and transmitted electron microscopy (TEM) can reveal the detailed microstructure of the collagen fibrils and vesicles where inorganic calcium phosphate is formed. For example, the striated appearance or the collagen fibril has a characteristic diameter of 67nm and length of 40-300nm (Woodhead-Galloway 1980). Furthermore, combined with positive and/or negative staining techniques, the periodicity of the fibrils can be very clearly revealed (Woodhead-Galloway, 1980; Hay, 1991;). Superstructure features of collagen can be easily investigated by TEM (Hay, 1991; Dubois et al, 1998). By using critical point drying techniques, SEM is a convenient method to observe the collagen fibres synthesised by osteoblast-like cells on biomaterials in situ. However, the shortcomings of EM and TEM for determination of collagen include: (a), specimen preparation is time consuming because very thin samples (<10μm) are required; (b), specimens could be damaged by electrons with high energy, resulting in artifacts; and (c), the inability to do quantitative measurements.

Polarised light microscopy may be another important method to investigate collagen fibres. Originally, polarised light microscopy was applied to investigate the structure and morphology of crystals and minerals. The particular advantages of the polarised light microscope lie in the fact that anisotropic effects reveal the presence of regular orientations of chemical bonds (Robinson and Bradbury, 1989). Therefore, the polarised light microscope is specifically able to investigate orientation in materials with anisotropic structure such as polymer fibres, collagen fibres (Hukins 1984), and cell walls (Lacey 1988). The preferred orientation of collagen fibrils in tissue section can be determined. Furthermore the strength and elastic feature, strain behaviour of collagen

fibres as well as other fibres can also be quantitatively determined by polarised light microscope (Lacey, 1989; Robinson and Bradbury, 1992; Oldfield 1994). Polarised light microscopy is not widely applied in biological research. In large part this is because polarising systems of adequately high resolution and sensitivity have not been available until quite recently. Till now, polarised light microscopy has not yet been used extensively to measure collagen fibres synthesised on biomaterials. Background materials, such as cell walls, microtubulin, fibronectin etc. with anisotropic structure may interfere with the imaging of the collagen fibres. Basically, microscopic analysis can only give qualitative but visual observation.

By combining the sensitivity and specificity of the antibody-antigen interaction, with the ability to visualise immuno-reactivity by microscopy, immunocytochemistry analysis is currently the most popular technique to determine collagen synthesised on biomaterials. Relying on the antigen-antibody specificity and sensitivity, one step or multi-step techniques may be selected. One-step methods use a labelled primary antibody directly against the antigen of interest; multi-step techniques use a primary antibody, followed by a labelled secondary or tertiary antibody. By multi-step techniques, the efficiency and sensitivity is improved. In all of these methods, the label serves as the marker for the site of the antigen. In practice, collagen antibodies labelled with fluorescent markers specifically combine with collagen (antigen), and after being scattered and excited by laser beam with certain wavelength, the fluorescent markers produce fluorescence which can be observed by CLSM. In order to be characterised as antibody-antigen specificity, immunochemistry staining must simultaneously fulfil two independent requirements: method specificity and antibody specificity (van Leeuwen, 1981). Both method and antibody specificity are crucial to the success of every immunochemical experiment. A positive-appearing result may be genuine, but there is a risk of non-specific or unwanted specific reactions which must be eliminated before the result can be accepted. Non-specific reaction may be produced by many factors.

Controls should be done to prove method specificity. One of these is to eliminate the primary antibody, and instead to incubate the cells or tissue in the antibody diluent alone. If any staining is retained when the primary antibody is omitted from the staining sequence, then such staining is non-specific and the reason may be that immunoglobulin with molecular structure similar to the primary antibody is present on the target tissue.

Another control is required to determine whether the tissue is natural autofluorescent. However, it is far more difficult to demonstrate antibody specificity than method specificity. Because of the nature of the antibody recognition, the antibody may not only bind to the antigen against which they were raised, but may also bind to similar molecules. Antibody non-specificity is commonly caused by any contaminating antibody in antisera, or by the binding of an antibody to molecules that are similar to the antigen. In order to check that a tissue is immunostained specifically, a negative control must be performed, in which primary antiserum is substituted by non-immunoserum or simply culture medium. All other conditions must be identical with those for the test. A positive control is essential and should be performed in parallel to testing materials. Without such a control, a negative control result on the test material will be meaningless, because there is no guarantee that the reagents are in good working condition and have been applied in the correct order and at the correct dilution (Polak and Van Noorden, 1997).

In our laboratory, for example, primary type I collagen antibody (Ab) followed by avidin+FITC, was applied to the antigen (type I collagen), and positive and negative controls were used to test method specificity and the antibody-antigen specificity. With the positive control, cells were cultured on both the material to be tested and standard cell culture slides and both Ab and avidin+FITC were applied to collagen that had been synthesised on the materials and slides. Two negatives were set up to ascertain that there was no reaction between type I collagen and avidin+FITC, and also that there was no fluorescence present in the antigen. Also cells were cultured on both the material to be tested and standard culture slide, omitting either Ab or avidin+FITC, when only Ab1 or only avidin+FITC is applied to the antigen (collagen), the results should be negative. If any staining occurs on negative control, non-specific binding sites should be blocked before applying the specific primary antibody.

Quantitative determination of collagen synthesised on biomaterials:

Electrophoretic separation techniques are extensively applied in biological research. Electrophoretic analysis separates proteins on the basis of the molecular weight of the protein (Chrambach et al, 1987). With regard to collagen detection, normally collagen fibre is degraded into protein chains followed by measurement of those protein chains by electrophoresis, then the amount of collagen could be calculated indirectly (El-Ghanam et al, 1997). During electrophoretic detection of proteins, two staining methods

are used: pre-electrphoretic stains and post-electrophoretic stains. By fluorescent stains (pre electrophoretic stains), as little as one nanogram of protein can now be detected (Chrambach et al, 1987). Coomassie Brilliant Blue Stains or Silver Stains are the most commonly used organic and inorganic stains for post-electrophoretic detection of protein separation. The important advantages of electrophoretic analysis of proteins include simple operation, proficiency and reproducibility. Many factors will influence the migration rate of protein during electrophoresis, for example, pre-electrophoretic stains may result in formation of covalent bonds with protein molecules, and consequently, alter the charge of proteins.

Choice of the techniques to determination collagen synthesised on the biomaterials:

With respect to biocompatibility evaluation via both in vivo and in vitro, the prominent advantage of EM, TEM, SEM techniques for collagen observation is to investigate the detail microstructure of collagen fibre in vivo and their important limitation is the difficulty to prepare specimens. Antibody staining followed by CLSM observation, radioactive isotope labelling followed by liquid scintillation counting (see Chapter **4**), and electrophoretic separation of proteins are extensively used to determine collagen synthesis on implant materials in vitro (Lemons, 1996; Dubois et al, 1998; Davies and Baldan, 1997; Garvey and Bizios, 1994). Before 1990, electrophoresis was the most important way to quantitatively determine collagen proteins but today both electrophoresis and radioactive isotope labelling are the most important methods to quantitatively determine collagen proteins synthesised on the biomaterials in vitro. Immunostaining followed CLSM observation is the most important method to qualitatively identify collagen fibre synthesised on the biomaterials in vitro model.

The aim of the present research attempts to quantitatively and qualitatively investigate the collagen synthetic activity of human and rat osteoblast cells on the calcium phosphate biomaterials and thereby to evaluate the material biocompatibility. Based on the facilities available in our department, I chose radioactive isotope labelling followed by liquid scintillation counting to quantitatively determine collagen synthesis on the materials, and chose antibody staining and CLSM observation as well as SEM observation to qualitatively determine collagen synthesis on the biomaterials at different culture times.

5.2 Materials and Methods

5.2.1 Preparation of materials

Dense and porous HA-Spinel and HA materials were prepared by the methods described in Chapter 2. Samples were 2.5 mm discs of 12 mm diameter. The surfaces of all samples were polished with 1200# silicon carbide paper, followed by cleaning in 70% alcohol and distilled water. Sterilisation of the samples was performed in an autoclave according to the normal procedure (121°C, 1.1 kg/cm^2 steam pressure for 30min) immediately prior to seeding cells on the samples. There were 5 kinds of materials used in these experiment, which were dense HA-Spinel (HS), dense HA, porous HA-Spinel with 24% porosity (PHS), porous HA with 26% porosity (PHA) and highly porous HA with 45% of porosity (HPHA). The pore size distribution ranged between 100μm to 500μm.

5.2.2 Collagen synthesis assessment

FFC cells, passage 28, at a density of 10^4 cells/per cm^2, and THO cells, passage 12 at a density of $2x10^4$ cells/per cm^2, were seeded on samples in 24 well culture plates, and incubated at 37°C, in an atmosphere of 5% CO_2 in air. One hour later, 1.5 ml culture medium was added to each well. The culture medium, DMEM, was used to culture FFC cells, and Ham's F10 was used to culture THO cells. The composition of the culture media was the same as described in Section **3.2**, and the culture media were renewed every second day. For comparison, the culture plate (polystyrene) was used as control material. After being incubated for 48 hours, both of the FFC and THO cells were treated with 37 KBq/ml L-(2,3-^{3}H)-Proline, in 1ml of the appropriate medium and incorporation of ^{3}H measured as described as in section **4.2.3.**

5.2.3 Collagen immunoflorescence analysis

THO cells were cultured on dense HA-Spinel discs and highly porous HA (with porosity of 45%) which were stuck to the bottom of 60 mm Petri dishes with sterilised medical adhesive tape, at a seeding density $2x10^4$ cells/per cm^2, and incubated at 37°C, in an atmosphere of 5% CO_2 in air. The culture medium was the same as above, and the medium was renewed every second day. At day 4, day 8 and day 14, the culture medium was discarded and the cells were washed with sterilised PBS 3 times followed by fixing

the cells on the material surface by the use of methanol-acetone solution with the ratio of 1:1. Samples were fixed over night at 4°C. 20µl of the primary antibody-Goat Anti-Type I collagen (Southern Biotechnology Associate, Inc., USA) which had been diluted in FCS at a ratio of 1:20 was applied to each sample in a small polythene box with a lid, lining the base of the box with tissue wetted with PBS. This was left at 4°C over night (18hr). After carefully washing the samples with sterilised PBS, 25µl of Neutralite Avidin-FITC (Southern Biotechnology Associate, Inc., USA) diluted 1:40 in PBS/BSA/Azide buffer (Composition of PBS/BSA/Azide is in appendix I) was applied to each sample and this was incubated in the wet box at room temperature for 3hr in the dark. The samples were washed 3 times with PBS again and mounted with mounting medium (Composition of mounting medium is in appendix III). The sample remained at 4°C, sealed down with nail varnish to prevent the cells dehydrating and wrapped up carefully in tin foil. Examination was performed by CLSM with a x100 oil immersion lens. Samples could be stored for a short time(over night at most) at 4°C in the dark. The conditions used on the CLSM for the observation of stained extracellular collagen fibres were as follows: excitation at 488nm, emission channel 1 and 2 at 590 and 530nm respectively, beam splitter at 510nm and the voltage of 700V. Two negative controls were set up to ascertain there was no reaction between type I collagen and avidin+FITC and also that there was no fluorescence present in the antigen. Cells were cultured on both the material to be tested and standard culture slide, omitting primary antibody-Goat Anti-Type I collagen on one control and avidin+FITC on the another control, i.e. only primary antibody-Goat Anti-Type I collagen or only avidin+FITC was applied to the collagen and the examination was performed under CLSM as described above to determine the specificity.

5.2.4 SEM examination

THO cells were cultured on the dense HA-Spinel and highly porous HA (with porosity 45%) materials, which were stuck onto the bottom of the 60 mm Petri dishes as before with the medical adhesive tape. Cells were seeded at a density of $10^4/cm^2$, and incubated at 37°C, in an atmosphere of 5% CO_2 in air. Culture medium was Ham's F-10 with the composition described as in Chapter 3. The culture medium was renewed every second day. The cells proliferated, migrated, and totally colonised the surfaces of both

dense HA-Spinel material and porous HA material. After 14 days and 21 days in culture, cells on the material were washed with sterilised PBS and fixed in 2.5% glutaradehyde (Omega, UK) in PBS buffer over night. The samples were washed in PBS buffer 3 times, followed by 3 washes in distilled water, and the specimens were dehydrated in a graded series of methanol prior to critical-point drying (CPD) (methanol-CO_2, using a Polaron apparatus). The graded series of methanol and treatment times are shown below:

Samples immersed in: 50% methanol, 2h;
70% methanol, 2h;
100% methanol, 2h;
100% methanol over night.

The disks with the cultured cells were mounted on aluminium stubs and coated with gold in a sputtering apparatus, and finally the specimens were examined at 15KV under a Jeol-840A Scanning Electron Microscope in our laboratory.

5.2.5 Statistical analyses

All measurements were collected and expressed as mean +/- standard deviations. Single factor analysis of variance (ANOVA) followed by Dunnett's test was employed to assess the statistical significance of results.

5.3 Results

5.3.1 Synthesis of collagen

The synthesis of collagen by both FFC and THO cells cultured on the dense and porous calcium phosphate materials is shown on Figure **5.1** The results demonstrated that there was a significant difference in the synthesis of collagen between cells cultured on the dense HA-Spinel materials and those cultured on the polystyrene control. This difference was apparent in both cell types and occurred to a similar extent in the FFC and THO cells. Collagen synthesis was decreased to 29.06% in FFC cells and to 33.87% in THO cells cultured on the dense HA-Spinel material compared with controls. For the THO cells the incorporation of ^{3}H-proline into collagen was 23587+/-6002 dpm when cultured on the polystyrene control material compared with 7991+/-2892 dpm on the dense HA-Spinel material ($p<0.05$, by ANOVA followed by Dunnett's test). With the FFC cells, the incorporation of ^{3}H-proline into collagen was 58650+/-15220 dpm when cultured on polystyrene control material compared with 17048+/-5760 dpm on dense HA-Spinel material. In contrast, compared with cells cultured on polystyrene, the cells cultured on HA showed no significant difference in collagen synthesis. THO cells cultured on the dense HA material incorporated 24403+/-5547 dpm of ^{3}H-proline into collagen and FFC cells cultured on dense HA material incorporated 59322+/-13503 In general, the synthesis of collagen was higher on the materials with greater porosity, and this was particularly evident with HA-Spinel material, although the trend was also observed with HA material especially with THO cells. The influence of the materials on incorporation of ^{3}H-proline into collagen was more readily observed with THO cells than FFC cells. Meanwhile, there were significant differences between the amounts of collagen synthesised on the dense HA-Spinel discs and the amounts of collagen synthesised on the porous HA-Spinel material discs.

Incorporation of ^{3}H-proline into collagen was two fold higher in FFC cells than in THO cells, and this was probably because FFC cells grow faster than THO and the cell number will be higher. The percentages of collagen protein in the total protein (measured by ^{3}H-proline incorporation) produced by THO and FFC cells on the materials are shown in Table **5.1**, and this was not significantly affected by the different materials.

Table 5.1. Percentage of collagen protein in total protein synthesised by human and rat osteoblasts on the calcium phosphate biomaterials. Results are mean +/-S.D., n=4.

Materials	**Percentage of collagen protein in total protein** (THO)	**Percentage of collagen protein in total protein** (FFC)
HPHA(45% porosity)	90.6638+/-7.97	92.99+/-6..83
PHA(26% porosity)	87.62+/-2.87	92.02+/-7.64
HA(<5% porosity)	91.55+/-4.51	85.70+/-7.25
PHS(24% porosity)	85.50+/-4.55	81.06+/-4.33
HS(<7% porosity)	89.04+/-1.08	89.03+/-5.31
Polystyrene	85.37+/-3.15	86.51+/-4.77

5.3.2 CLSM investigation of collagen fibres by immunochemical staining

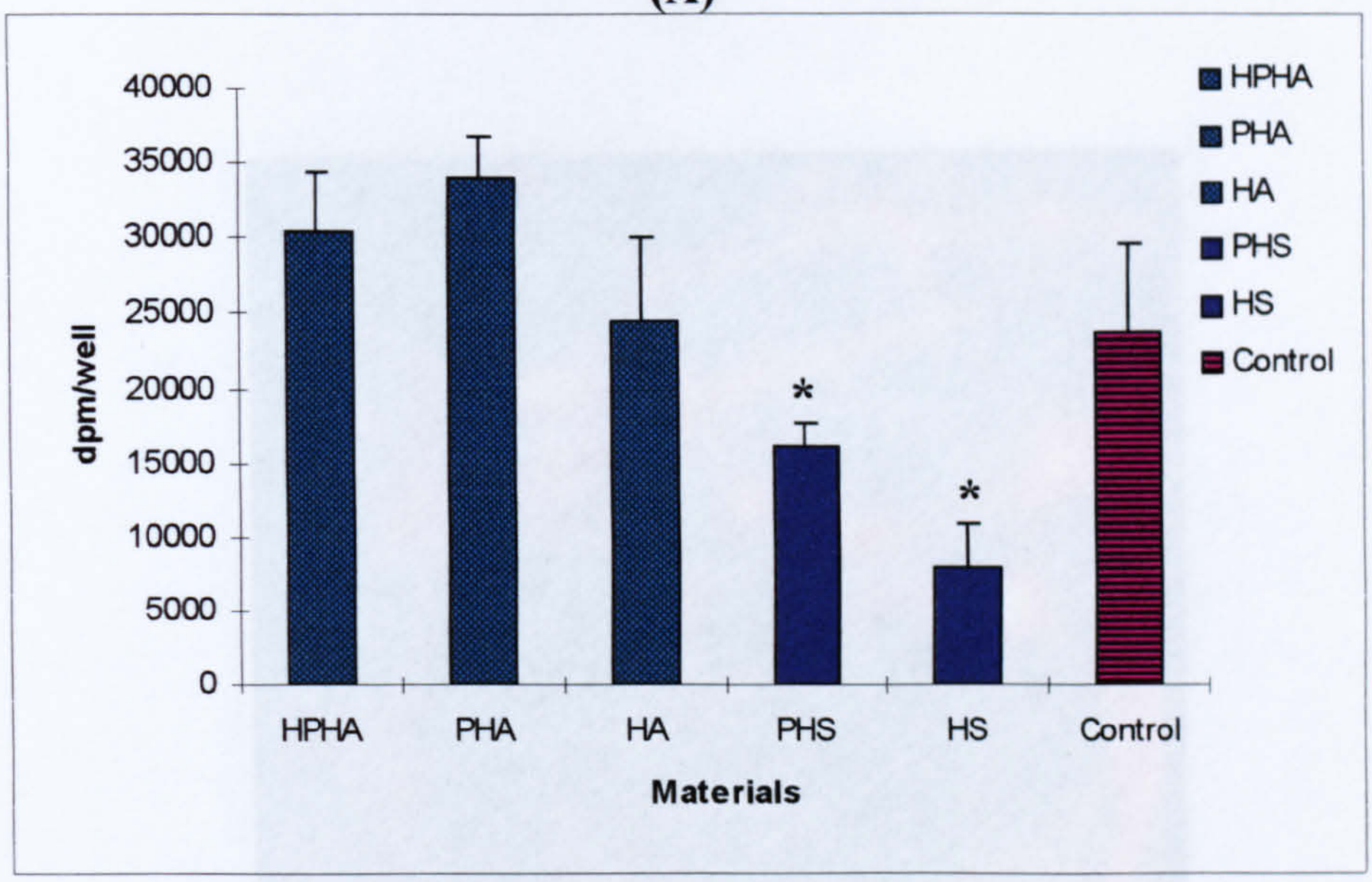
Negative control examinations by CLSM showed that there was no reaction between avidin+FITC and type I collagen, and also, without avidin+FITC labelling, the reaction between antibody and antigen cannot be directly observed under fluorescence microscopy on either the material to be rested or the slide. By staining of the type I collagen with FITC labelled antibody, CLSM observation demonstrated that type I collagen had been synthesised by THO cells on dense and porous material surfaces. The morphology and structure of the collagen fibres synthesised by THO cells are shown in photograph **5.1-5.2**.There were no collagen fibres found on the material samples where THO cells were cultured for only 4 days. After 8 days in culture, type I collagen was produced by THO cells and formed a network structure surrounding the cell body on the dense HA-Spinel discs. Compared with the type I collagen network formed on dense HA-Spinel material discs, the collagen fibres formed on the porous HA discs were longer but less dense. Formation of collagen fibres may be related to the rough surface of the porous material. After 14 days in culture, the density of the collagen network produced by THO cells was increased.

5.3.3 SEM examination

Photograph **5.3** shows the THO cell morphology and extracellular matrix fibres formation on the dense HA-Spinel biomaterial on which THO cells have been cultured for 21 days. Cells formed multilayers on the surface of the dense HA-Spinel, and nodular structures where mineralisation occurred can be seen, connected to the cell surface by

extracellular fibres. In certain zones, needle-like extracellular fibres were found to connect the cell bodies. However, on the material surface without the presence of cells, there was no evidence for HA crystal formation on the dense HA-Spinel material. SEM images of porous HA seeded with the THO cells after 21 days in culture are shown in photograph **5.4**.The same multilayer cultured cell structures and nodules covered the surface of the porous HA material. In certain zones, on the porous material where cells had not grown, fine HA crystals were found by EDAX. EDAX determination is convenient to identify the newly formed HA crystal. This is in contrast to the dense HA-Spinel material where no fine crystal structures were observed in the present research. The results show that a chemical reaction has taken place on the porous material during cell culture.

(A)



(B)

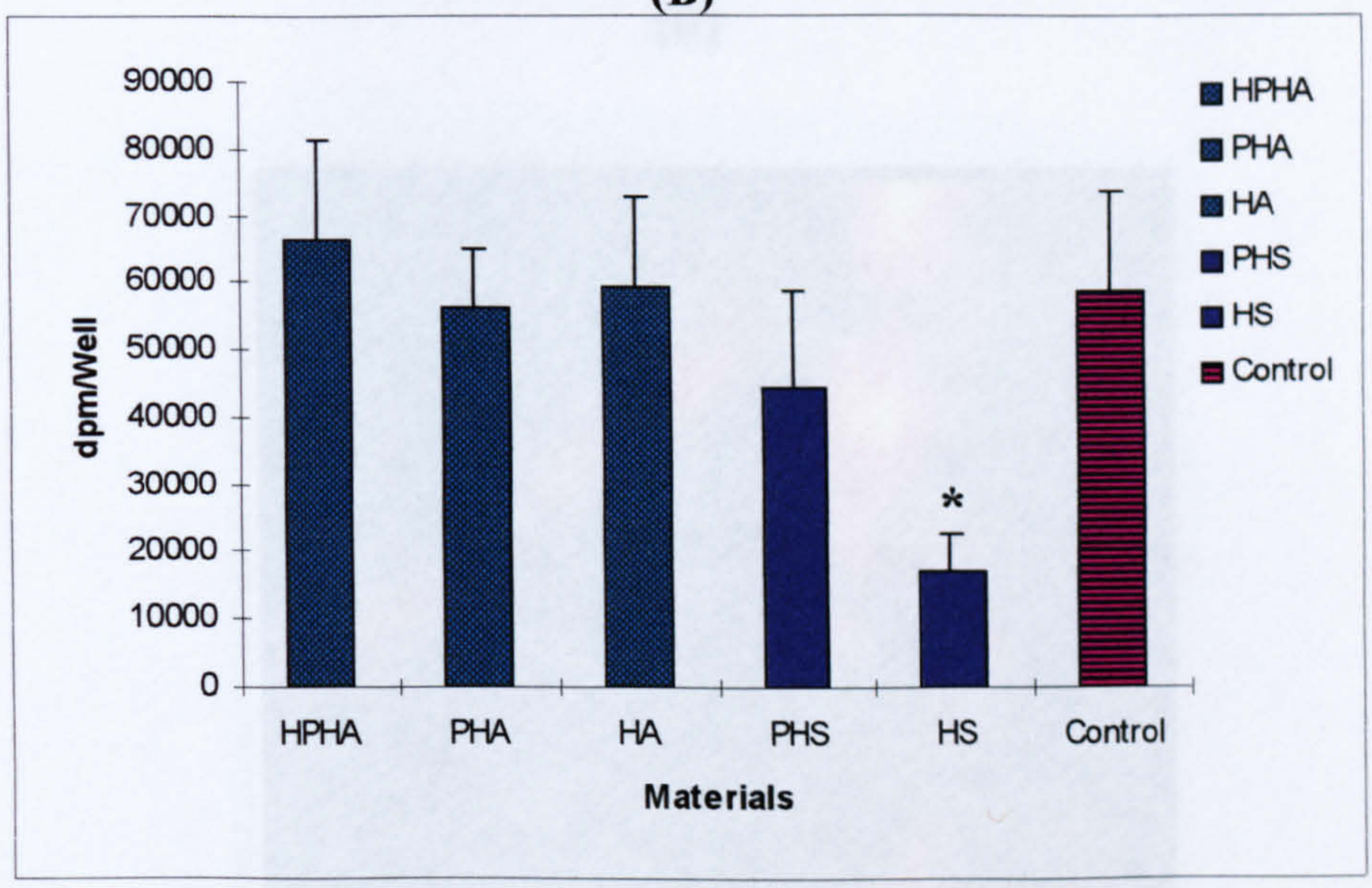
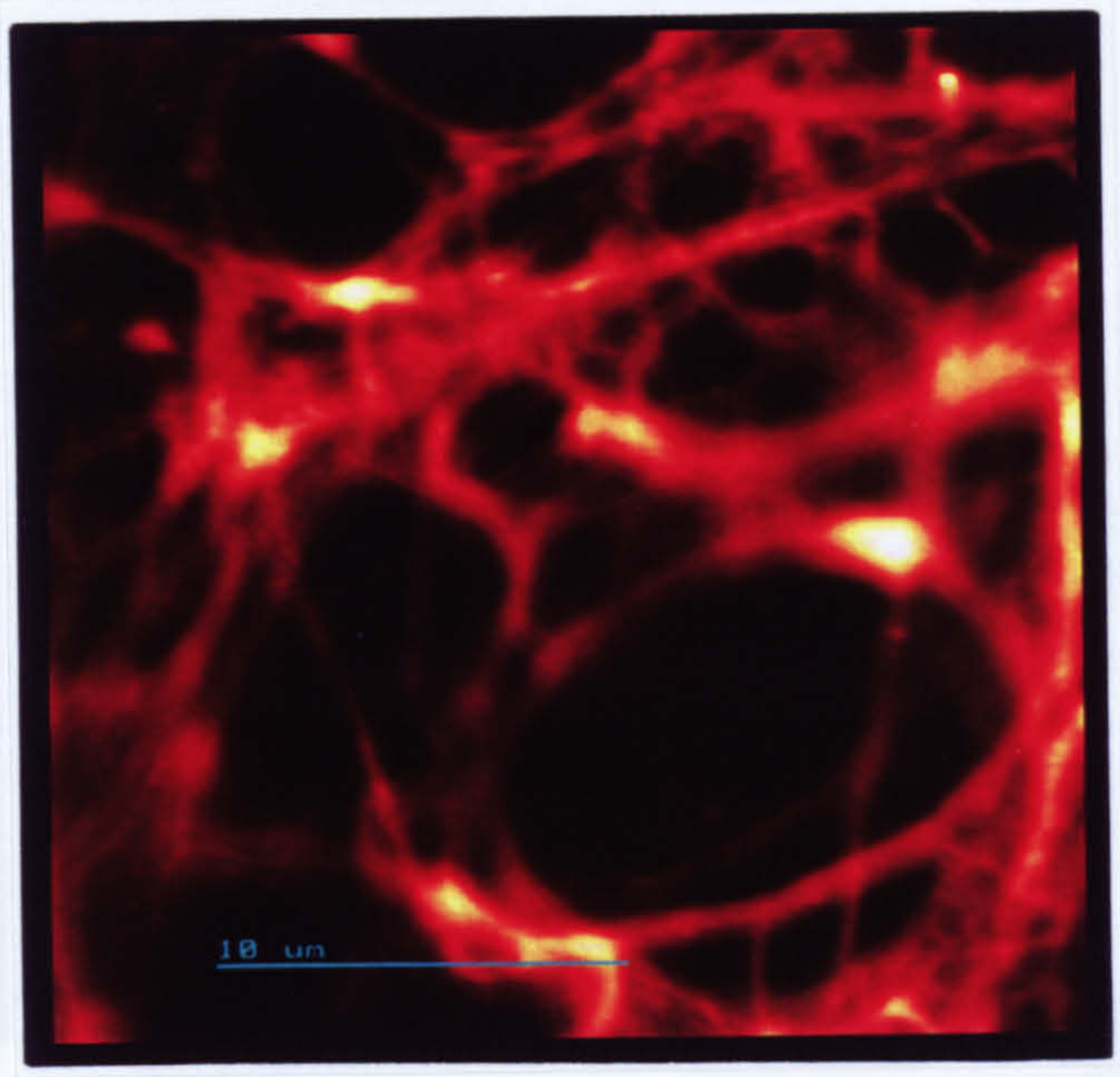
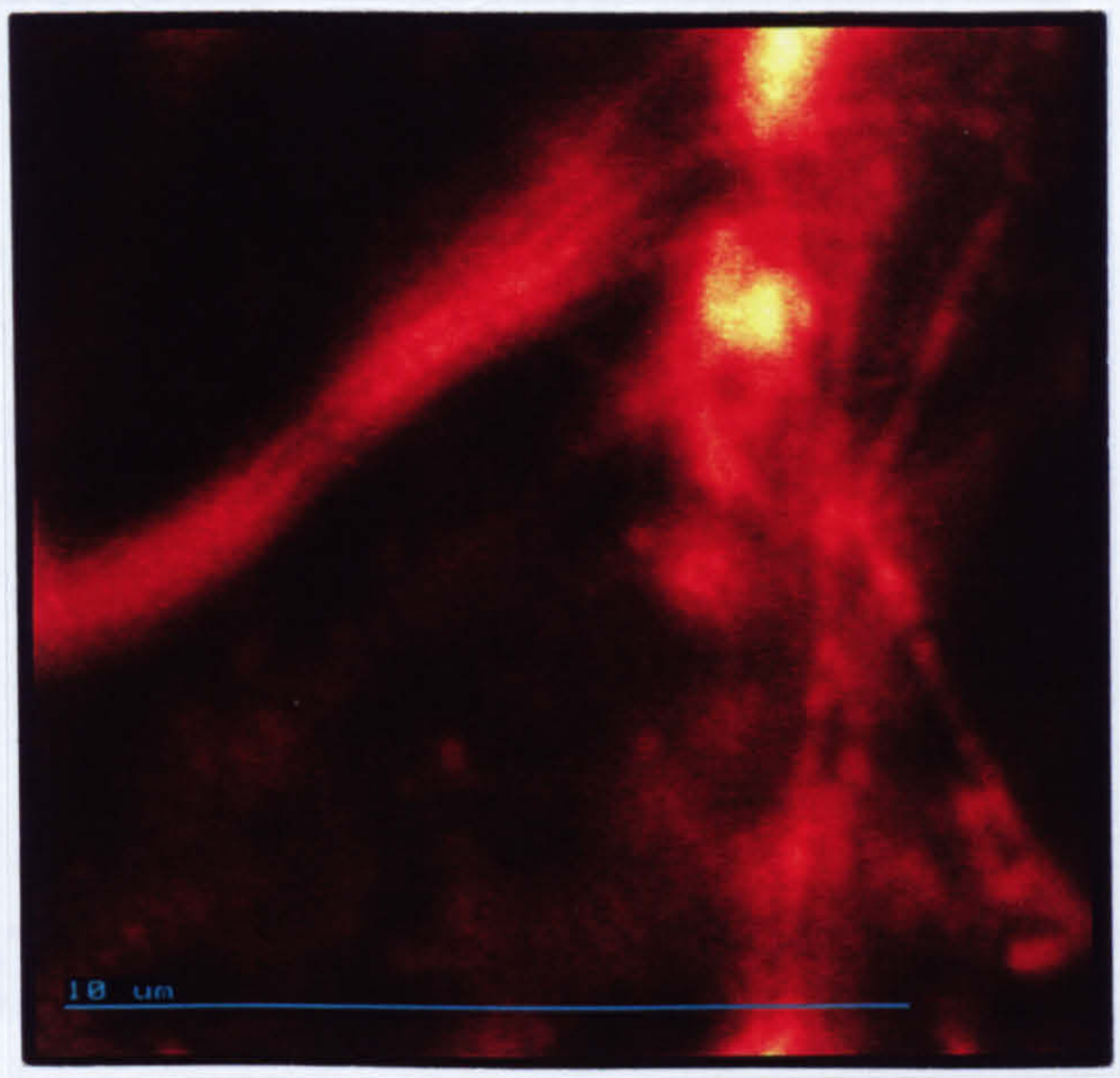


Figure 5.1 Incorporation of ^{3}H-proline into collagen by THO cells (A) and FFC cells (B) cultured for four days on porous and dense HA materials and HA-Spinel materials. HPHA: HA, porosity, 45%; PHA: HA, porosity 26%; HA: HA, porosity<5%; PHS: HA-Spinel, porosity 26%; and HS: HA-Spinel, porosity5-7%. Cultures were initiated in 24-well plate at a seeding density of $2x10^4/cm^2$ for THO cells and $10^4/cm^2$ for FFC cells. Results are mean +/- SD mean, n=4. *P<0.05, compared with control by the use of ANOVA followed by Dunnett's test.

(A)

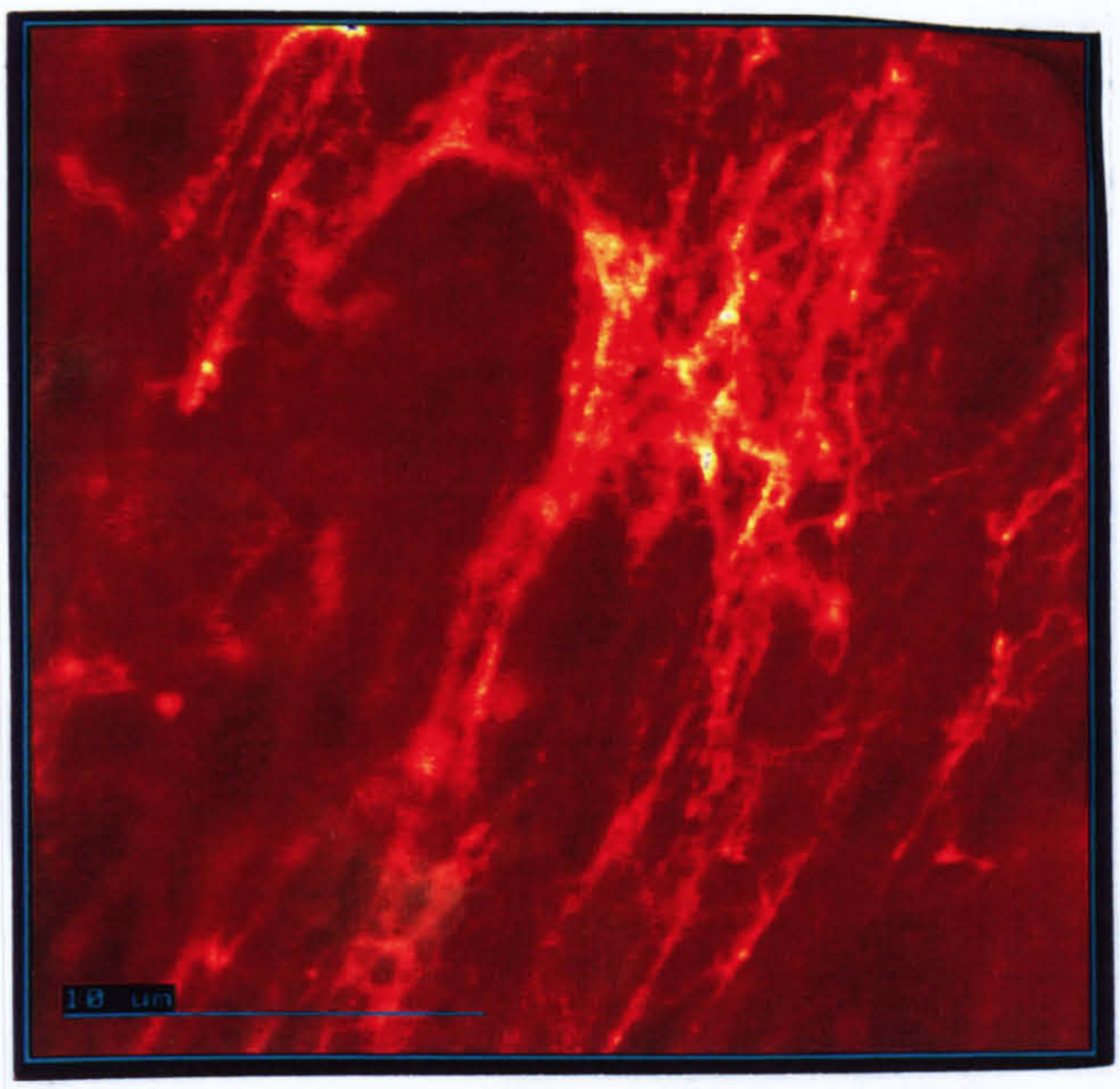


(B)

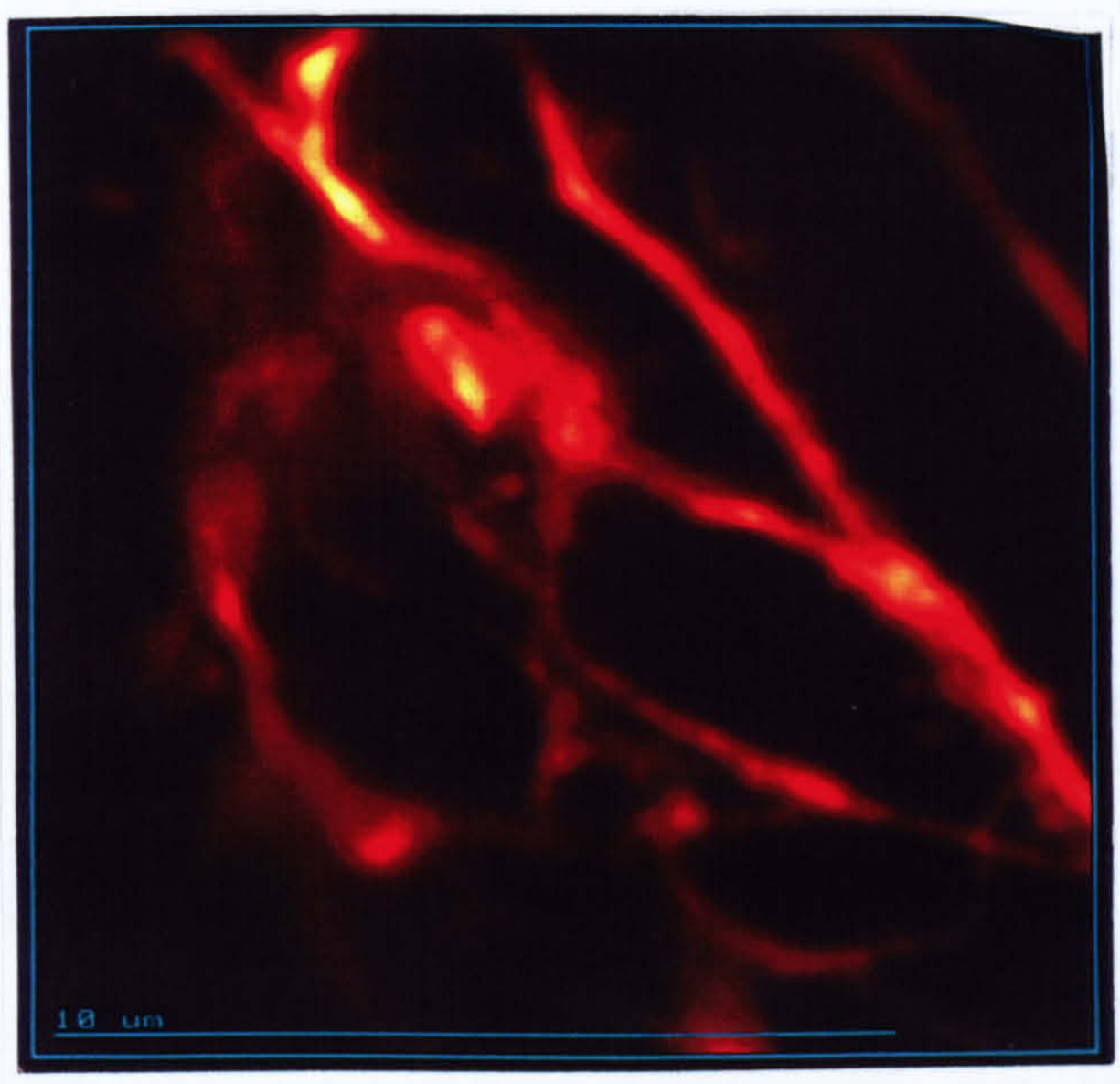


Photograph 5.1. Immunofluorescence staining of type I collagen fibres produced by THO cells. (A) Day 8: a network of type I collagen fibre can be seen on the HA-Spinel background. (B) Day 8: detail structure of type I collagen fibres on HA-Spinel. Scale bars have been shown in photographs. Stained by goat anti type I collagen antibody, followed by avidin+FITC labelling. CLSM observation (excitation at 488nm, emission channel 1 and 2 at 590 and 530nm respectively, beam splitter at 510nm and the voltage of 700V). Imaging area: 28x28μm (A), 13x13μm (B)

(A)

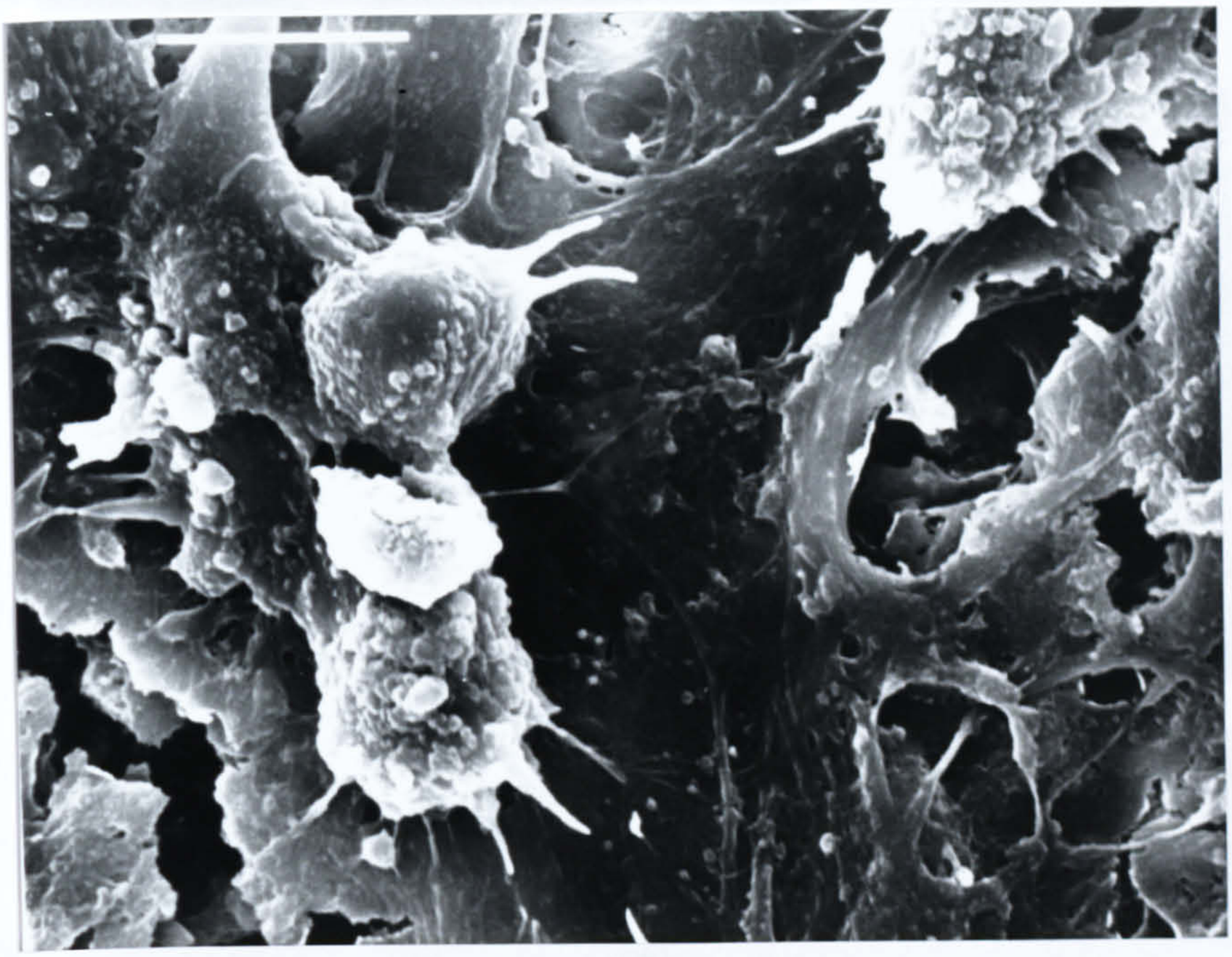


(B)

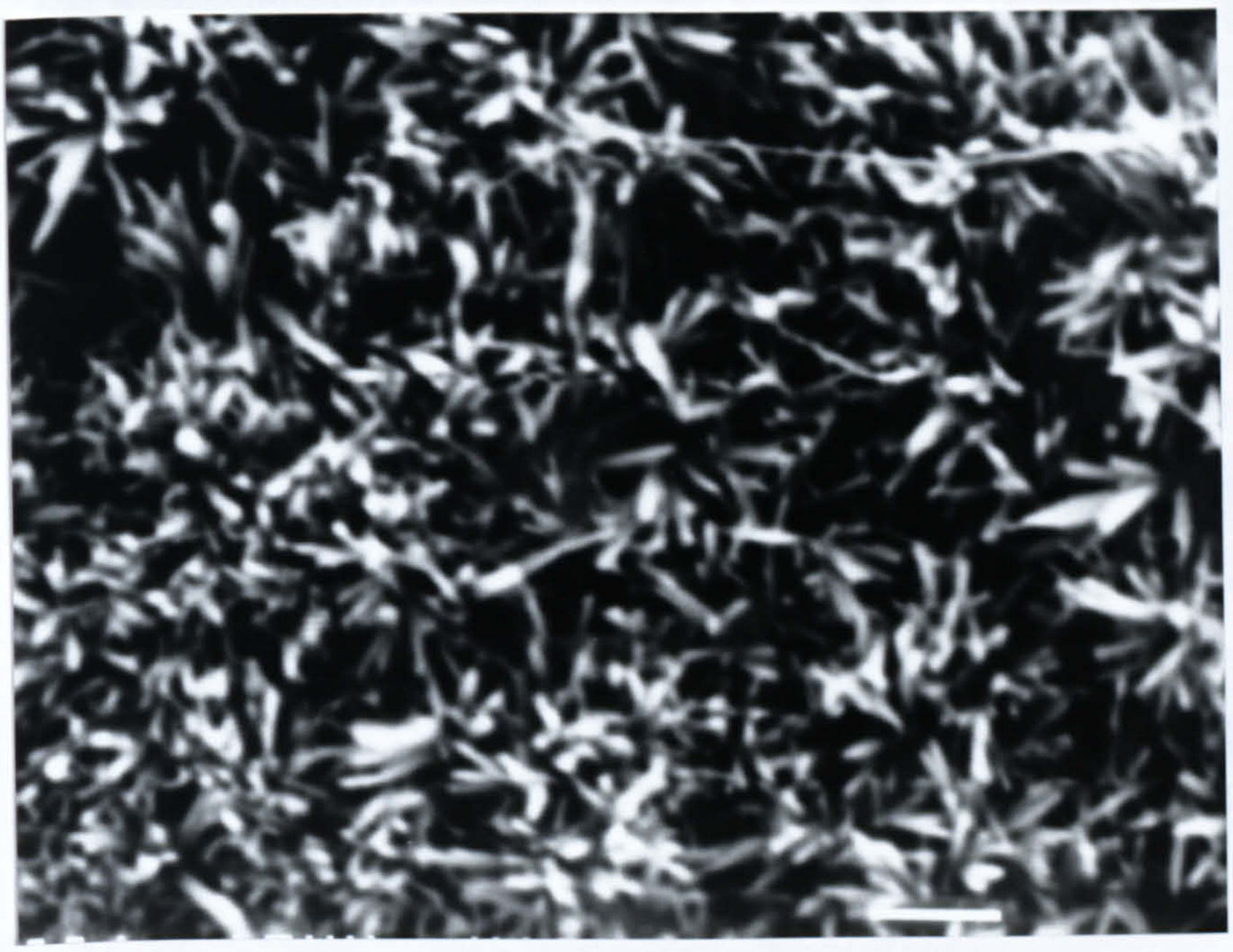


Photograph 5.2. Immunofluorescence staining of type I collagen fibres produced by THO cells. (A) Day 14: a dense network of type I collagen fibre can be seen on the HA-Spinel background. (B) Day 8: Type I collagen fibres can be seen on the porous HA material surface. Scale bars have been shown in photographs. Stained by goat anti type I collagen antibody, followed by avidin+FITC labelling. CLSM observation (excitation at 488nm, emission channel 1 and 2 at 590 and 530nm respectively, beam splitter at 510nm and the voltage of 700V). Imaging area: 28x28μm (A), 13x13μm (B)

(A)

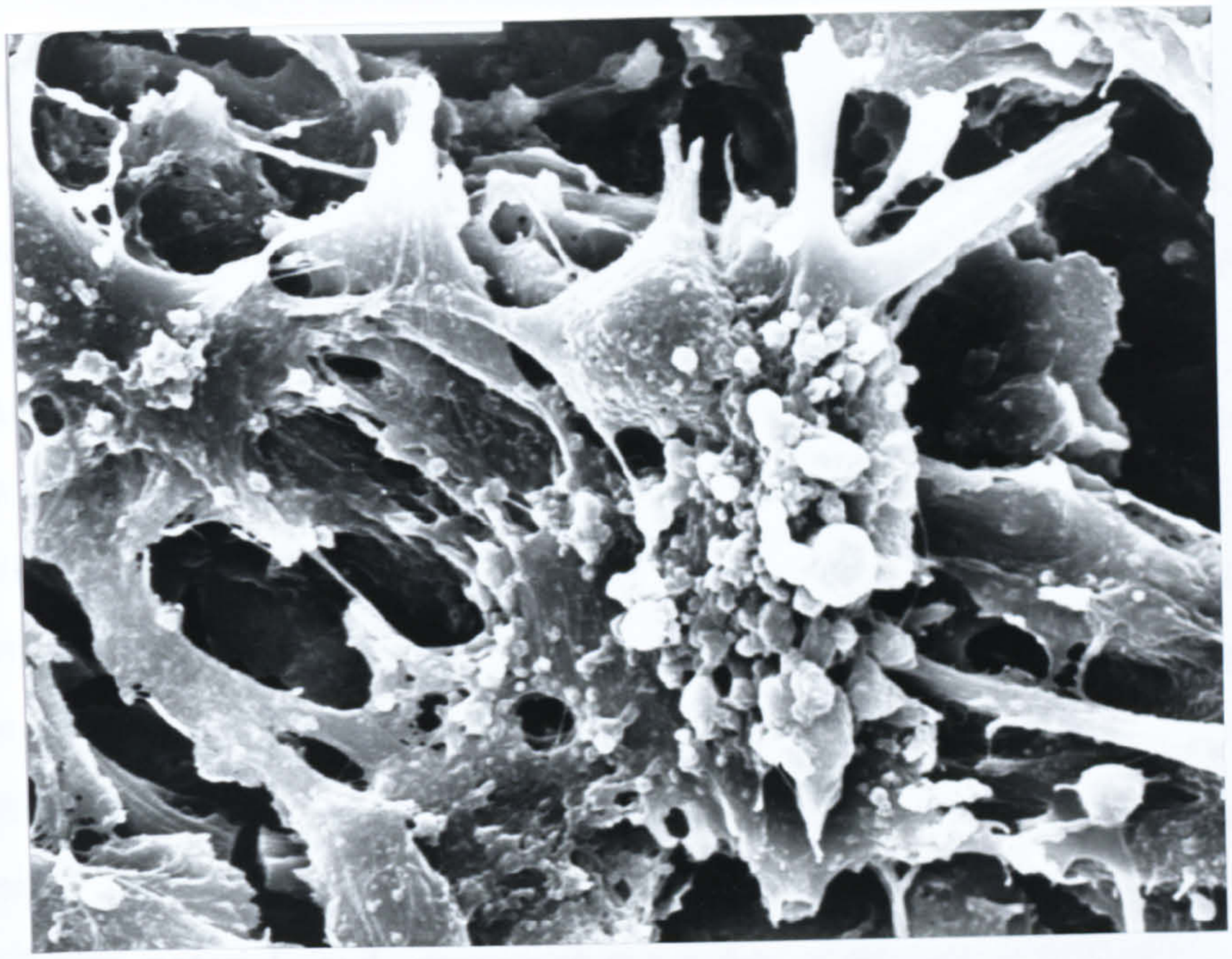


(B)

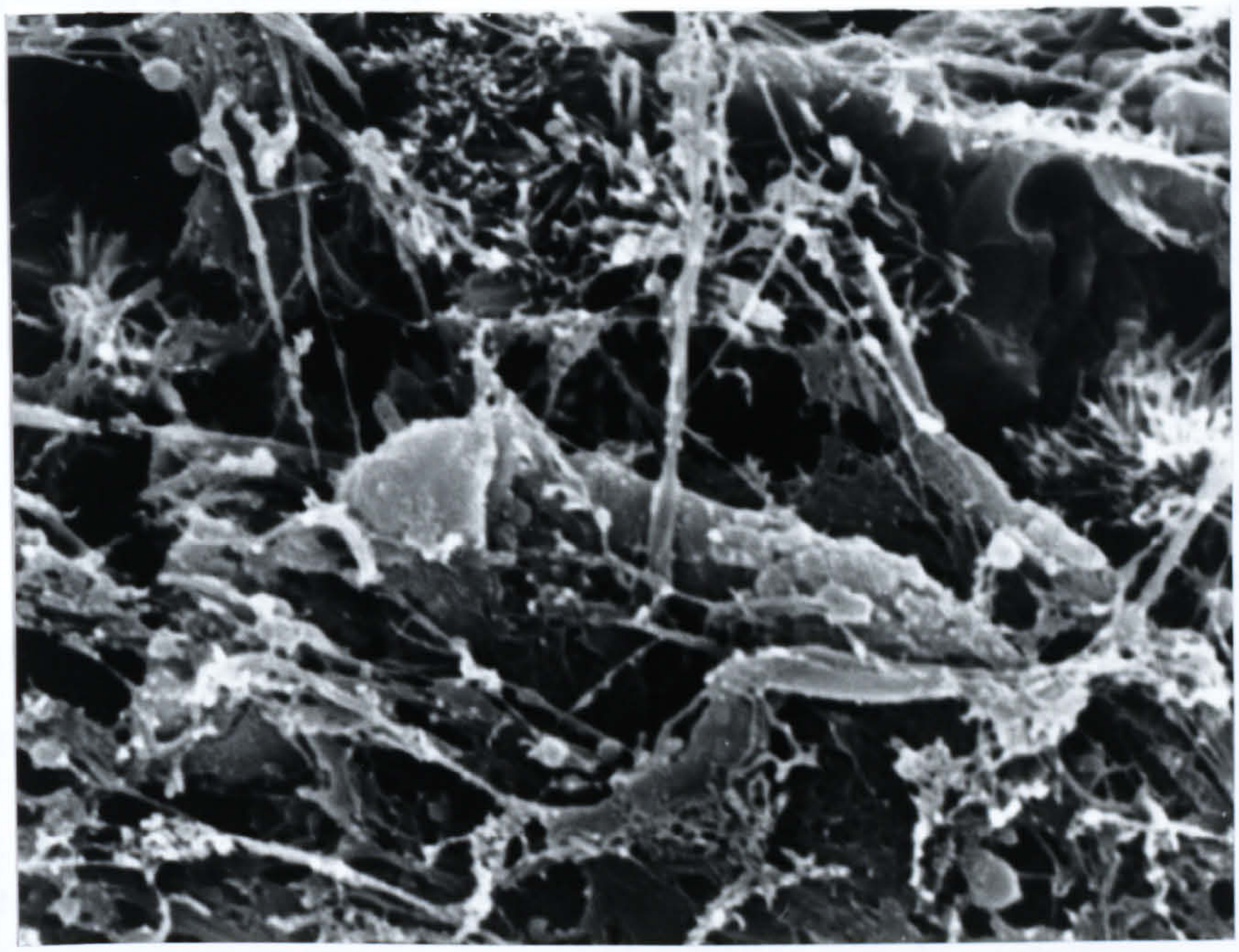


Photograph 5.3. SEM (15kv) images of THO cells cultured on dense HA-Spinel discs for 21 days. The multilayer structure of cells and the nodules on the cell bodies are shown in (A) and the presence of extracellular fibres which connect cell bodies is shown in(B). Magnification: x4000 (A) and x7500 (B). Imaging area: 60x52μm (A), 30x26μm (B)

(A)



(B)



Photograph 5.4 SEM (15kv) images of porous HA material (porosity: 45%) with THO cells after 21 days in culture. (A); The porous HA material disks were covered with multilayered cells and (B); High magnification showing that HA crystals were formed on the material surface on areas of low or absent cell growth, with crystal size about 2 μm. Magnification: x4000 (A) and x15000 (B). Imaging area: 60x52μm (A), 15x12.5μm (B)

5.4 Discussion

The research results in the present chapter demonstrate that human osteoblasts synthesise collagen on both dense HA-Spinel and porous HA materials. The amount of collagen synthesised by cells cultured on the porous HA sample is much higher than that of cells cultured on the dense HA-Spinel and porous HA-Spinel samples. It is also higher than the amount of collagen synthesised on cells cultured on the dense HA sample. In my opinion, it is related to the large surface area of the porous materials. Compared with the dense materials, within a limit of porosity and a range of pore sizes, with the increasing surface area in the porous materials, the distribution area of the cells is increased, and as a result the area available for cell attachment and proliferation is increased, and consequently, the amount of extracellular matrix formed by osteoblasts is increased. Similar results have been reported by Ishaug (1997) who cultured rat osteoblasts on porous polymer materials and measured the depth of the mineralisation in the porous materials. In fact, apart from the material composition, the most important difference presented between dense materials and porous materials is that porous materials supply more area for cells attachment, and supply more volume for deposition of extracellular matrix. Meanwhile the rougher surface of the porous materials is better suited for cell anchoring and attachment, and this has been the concern of much research. Bagambisa and Joos (1990) stated that osteomorphogenesis is closely associated with the adhesion, anchorage and spreading of cells, and that the formation and deposition of bone directly onto an implant requires the surface to be not only non-toxic, but also to favour this behaviour. Various factors would affect the differentiation of osteoblast like cells, and consequently, affect the bone forming process. The influence of surface topography on the bone integration of implants is a perfect illustration of this (Bagmbisa and Joos, 1990).

The physical characteristics of rough-texture and porous-coated titanium surfaces both enhance the cellular adhesion and subsequent mineralised matrix production (Knabe et al 1997; Groessner and Tuan, 1992; Buser et al, 1991). Studies in vivo also found that rough surface (pore size in the range of 2-50μm) can produce better bone fixation than smooth surface (Carlsson et al, 1988; Wilke et al, 1990). By implantation experiments in miniature pigs, Buser et al (1991) found that the rough surface (pore size in the range of

40-110μm) of HA-coated titanium implants generally demonstrated an increase in bone apposition compared with the polished fine structured surface of titanium or plastic material. In fact, compared with the electropolished surface, the rough textural surface can supply more contact area or anchoring sites for cells. The research of Groessner and Tuan (1992), who cultured chick embryonic calvarial osteoblasts on smooth, rough textured (with paralleled grove size: 5-25μm) and porous-coated titanium surfaces (pore size in the range of 5-45μm), revealed that osteoblasts adhered securely to Ti surface and frequently bridged the uneven surface by means of cellular processes. The extracellular matrix formation and subsequent mineralisation were both substantially enhanced in the culture on rough-textured and porous-coated titanium. They strongly suggested that a porous or rough titanium implant surface may act like a natural substrate for tissue/cell ingrowth. Furthermore, Ishaug et al (1997) demonstrated that rat stromal osteoblast proliferation and function were not affected by polymers of pore sizes in the range of 150-710μm. The adhesion of anchorage-dependent cells is the first step in the process of cell-surface interactions and it has especially critical importance for cell growth, proliferation and differentiation (Hunter et al, 1995;Okamoto et al, 1998). A rough (pore size in the range of 1-20μm) surface is more suitable for human osteoblast-like cells (MG63) anchorage and attachment, and has stimulatory action for osteoblast-like cell proliferation as well collagen synthesis (Martin et al, 1995; Goldring et al, 1990). Okamoto et al (1998) demonstrated that RGD peptides on the membrane regulate the specific adhesion of human osteoblasts to HA ceramic but not to titanium alloys. Protein synthesis and matrix production were also surface -sensitive. Inhibition was observed on the smoothest titanium. In contrast, RNA synthesis was enhanced in cell cultures on rough surface with pore size ranged 5-45μm (Martin et al, 1995). Faucheux et al (1994) demonstrate normal proliferation and collagen synthesis of human osteoblast like cells on HA with pore size in the range of 150-750μm. Fibronectin, a cell adhesion protein in serum, has been shown to mediate cell attachment and spreading on artificial substrates by interacting with glycosaminoglycans and the cytoskeleton. It is possible that the rough surface may have adsorbed more fibronectin than the smooth surface, preserving the synthesis of extracellular matrix protein(Martin et al, 1995; Grinnel and Humphries, 1986).

There are two general processes by which mineralisation can be inhibited: (1) by the removal and/or degradation of HA inhibitors at a particular site and (2) through the presence of HA nucleation that promotes HA crystal formation and growth by providing alternate low activation energy pathways of HA nucleation despite the unaltered presence of inhibitory molecular species. Dubois et al (1998), Lemons (1996) and Sautier et al (1991) demonstrated that HA biomaterials and active glass biomaterials have a stimulation action of the formation of extracellular matrix as well as on the formation of HA crystals. In the present research, the collagen synthesis of both human and rat osteoblasts cultured on dense HA material is similar to that of cells cultured on the control material (polystyrene culture plate). The results demonstrated that both HA materials and the culture plate material have similar effects on the collagen synthesis of both human and rat osteoblasts. However, the amount of type I collagen synthesised by osteoblast cells cultured on either the dense HA-Spinel material, or the porous HA-Spinel material was much less than that of cells cultured on both the HA material and the control material. This implies that the Spinel ($MgAl_2O_4$) in the HA-Spinel materials has an inhibitory effect on the formation of type I collagen. The inhibitory mechanism is not clear at present. It has been suggested that the Al and Mg ions present have an effect on the proliferation rate and/or the cell differentiation function. One factor that may impair collagen synthesis on HA-Spinel is that Spinel is a bioinert material. It is commonly considered that no osteointegration is performed at the interface between bioinert materials and bone tissue and that a soft tissue capsule is formed surrounding the inert materials for insulating the materials. In contrast to my results, Gregorie et al (1995) demonstrated that the amount of type I collagen synthesised by MC3T3-E1 on the HA powders and TCP powders was significantly higher than that synthesised on the control material (tissue culture plate). In the present research, after being combined with Spinel, the bioactivity of HA was limited partially, and consequently, both cell number and the amount of collagen synthesised on the HA-Spinel composite have decreased.

Al ions in solution in vitro slow the direct precipitation of HA, and slow the transformation of amorphous calcium phosphate (ACP) to HA. In vitro studies suggest a significant physicochemical inhibitory effect on HA formation. Heimke (1992) stated that Al^{3+} can induce cellular changes in osteoblast function and possibly exerts an effect on cell metabolism. Proteoglycans can delay HA and c-HA crystal formation, because large

volumes of solution containing enormous macromolecules become unavailable for HA formation and growth. Mg ions enter the structure of the forming HA nuclei by replacing Ca, resulting in a distorted atomic structure that slows subsequent growth to HA. Al ions delay HA formation, not by entering the structure of forming HA nuclei, but by adsorbing on the surface of growing HA crystals. Serum proteins slow the transformation of amorphous calcium phosphate(ACP) to HA by adsorption on the ACP surface, which decreases its dissolution rate (Blumethal 1989).

In my present research, after 8-21 days culture, on both porous HA and dense HS biomaterial, extracellular fibres and mineralised nodules have been produced by the THO cells. The morphology of THO cells cultured on both dense HA-Spinel and porous HA biomaterials as well as the fibres were similar to those described for rat osteoblast cells (MC3T3-E1) cultured on porous bioactive glasses and porous HA by El-Ghannam (1997) who proposed that the fibres are collagen fibres. Usually, mineralised nodule formation is considered to be the final step of differentiation. An important marker of mineralisation of osteoblasts on implant materials is that nodules of various sizes and shapes are produced by the cells. According to Lemons (1996) and Neffussi (1985), the cells had synthesised collagen matrix in these nodular structures where mineralisation occurred. In the central part of the nodules, the cells were surrounded by collagen matrix, matrix vesicles, and minerals. By SEM examination, we found many nodules of various sizes and shapes on the cell multilayer. The morphology of extracellular matrix and THO cells in the present research were similar to those of nodules observed by El-Ghannam (1997) who cultured rat osteoblast cells (MC3T3-E1)on porous bioactive glass and Nefussi (1985) who cultured rat osteoblast cells on polymer material. Dubois et al (1998), by the use of TEM observation, demonstrated that the nodule structure was the place where mineralisation was carried out. According to Dubois and Nefussi, cells synthesise collagen matrix in these nodular structures, and consequently, mineralisation is carried on in these nodular structures. Cells are surrounded by collagenous matrix, matrix vesicles, and minerals. It should be noted that as observed by SEM, the whole surface of the porous HA material was not covered with mutilayered osteoblast cells. On some areas, apart from the newly formed HA crystal, no cells were found, and the surface of the pore was smoother, like fused glass. The rough surface of the porous material may result in an uneven distribution of cells. Also some cells may migrate into and grow in the

deep pores, therefore, the cell distribution on the porous material surface was different from place to place.

After immunofluorescence staining, by CLSM observation, I did not find type I collagen fibres formed after 4 days culture of THO cells either on the dense HA-Spinel sample or porous HA. The 4 day period was too short to have resulted in measurable collagen fibre production. Collagen fibres were found on the day 8 samples and day 14 samples. Despite much study, the exact spatial and temporal relationships of HA nucleates and inhibitors in calcifying tissue are still poorly understood. Collagen is certainly a crucial site of mineral regulation and deposition as evidenced by the location of apatite crystals within collagen fibrils and on their surface. In addition, extracellular membrane-bound vesicles have been proposed as the site where some mineralisation is initiated and controlled. A considerable number of non-collagenous proteins function as regulatory molecules for mineralisation, calcium modulation, cell regulation, and bone regeneration. In my experiments, collagenous protein synthesised by THO cells and FFC cells on both porous and dense materials was approximately 80-92% of the total proteins synthesised by cells as measured by incorporation of ^{3}H-proline. By antibody staining and SEM examination, the research results clearly demonstrated the formation of type I collagen on HA-Spinel and HA crystals formed on the porous HA material. In contrast to my research results, Nefussi et al (1985) demonstrated that when the culture medium was supplemented with β-glycerophosphate, collagen fibres were produced by rat osteoblast after 4 days culture. This was dependent on the presence of increased phosphate concentration in the culture medium and their experiment also demonstrated that osteoblast cells grown in the absence of β-glycerophosphate do not show collagen formation during such a short period of culture. According to the suggestion of van der Rest (1991), collagen undergoes a remarkably extensive post-translational processing. Before the triplet Gly-X-Ys are finally crosslinked together, the procollagen would be formed firstly in cells and secreted out through the cellular membrane. In the present research, although type I collagen has been measured by radioactive isotope analysis on the cells in four days culture, immunochemical staining shows that no collagen fibre was found on the samples where cells cultured for four days. We suggest that the type I collagen measured by radioactive isotope analysis was the collagen precursor, procollagen, and that 4 days in culture, was too short a time to observe collagen fibres

production by our CLSM. The testing results of controls showed that the testing materials did not react to avidin+FITC and there was no autofluorescence in materials.

It is unclear where the HA crystals on the porous HA come from. One possibility is that the new HA crystal formation was mediated by the culture medium, produced via the path of precipitation-deposition. Another possibility is the new HA crystal formation was mediated by cell differentiation. I prefer the former situation. Mg has a considerable inhibitory effect on the formation and growth of HA when Mg is either co-precipitated with amorphous calcium phosphate (ACP) for preparative solution, or placed in solution. The time required for ACP to be converted to HA is increased and the transformation rate is decreased(Kevor et al, 1997). In blood serum, the Mg/Ca ratio is about 0.3, and by itself, this is sufficient to prevent HA precipitation in that blood fluid. The harmful effect of the chemical properties of a material is not always accompanied by cell morphological and ultra-structure modifications, but it can alter either cellular energy metabolism or protein synthesis. Osteogeneic cell cultures exposed to Ti^{+4}, Al^{+3} ions, and Ti-6Al-4V alloy showed no cell number decrease, but did show an almost complete suppression of osteocalcin secretion and mineralisation (Itakura et al, 1998; Tompson et al 1995). The normal (nonpathologic) mineralisation process in the body is controlled by complex physicochemical and cellular regulation of substances that promote and inhibit HA formation. Simulated body fluids (SBF), such as plasma, are supersaturated with respect to HA, and the question of why humans do not mineralise all over and literally turn to stone arises. The answer is that the significant concentration of Mg ions and protein in plasma effectively inhibit HA precipitation in tissues exposed to that body liquid. These observations suggest that Spinel ($MgAl_3O_4$) in the dense HA-Spinel material may impair HA crystal formation on the material. Based on these reasons, therefore, HA crystals were not found on the dense HA-Spinel discs in the present research. If Al^{3+} and Mg^{2+} ions are released from the material surface, they may disturb the expression of osteoblastic phenotype and cellular function (Itakura et al 1998) and release of these ions may account for the lower collagen synthesis observed on the HA-Spinel materials.

Chapter 6 Protein adsorption on calcium phosphate bioceramic and surface modification via immersion

6.1 Introduction

Surface modification is a complicated physicochemical and biochemical process, which includes organic and inorganic reactions between the immersed materials and immersing media, such as protein adsorption onto the materials, ion release from the material surface to the immersing media, or ion precipitation onto the material surface. The events alter the physical microstructure and composition of the material surface and consequently modified surfaces are available for cell attachment, differentiation and proliferation. Protein adsorption from the media onto the biomaterial surface, and its ability to stimulate the interaction between implant and tissue, depends on the characteristics of the materials, such as its chemical composition, surface condition, porosity and density, and even crystal size. Accompanied by protein adsorption onto material surfaces, physicochemical reactions take place at the solid-liquid interface, to form bioactive equivalent carbonated apatite(c-HA). Bone bonding between implant and living tissue relies on the formation of c-HA.

6.1.1 Protein adsorption onto biomaterials

Protein adsorption onto biomaterials aims to modify the material surface, and then to enhance cell anchoring, and proliferation on the materials. The method of protein coating is rather simple. Commonly, the biomaterials are immersed in the medium with protein for a period of between minutes to several days. Modifications of the microstructure as well as the composition of the material surfaces results in the improvement of the biocompatibility of material surfaces(Anselme et al, 1997; Martin and Brown, 1994). Usually apart from protein deposited onto the material surface, some other chemical reactions between the media and immersed block will also take place. The important media most often used include: stimulated body fluid (SBF), cell culture media supplemented with serum, plasma, distilled water, and deionsed water (DI water), etc.

Proteins adsorbed often involve fibronectin, fibrinogen, albumin, vitronectin, plasma glycoprotein, IgGγ, IgD,IgM, etc. (Martin and Brown, 1994). At present we are

still not able to give an accurate measurement of the amount of the above proteins present on the material surface in moles or in grammes. In general, albumin adsorption is higher than that of the other proteins when medium supplemented with serum is used (Luck et al, 1998). The effect on the cells is assessed by ALP activity, collagen synthesis, cell orientation, distribution, attachment, and proliferation, carbonate apatite formation. Changes in the structure of the material surface are usually analysed by fourier transformed infrared spectroscopy(FTIS), X-ray, SEM, TEM, etc. Changes in Ca^{2+}, and PO_4^{3-} content of the immersing media are measured by flame atomic absorption spectroscopy and phosphorus concentration is measured by colorimetric detection of phospho-molybdate respectively (Brunstedt et al, 1993).

Cell adhesion on biomaterials is enhanced by mainly modifying the surface with serum protein, albumin, fibronectin, vitronectin and collagen. Mammalian sera contain two major cell-adhesive proteins, fibronectin and vitronectin. Thomas et al (1997) provided evidence that initial adsorption of vitronectin may lead to enhanced bone-derived cell mineralization on implant materials. Adsorption of serum protein can effect the focal contacts and the cytoskeletal arrangement of the cells which subsequently affects cell morphology and function. Through receptor-ligand interaction, the cytoskeleton receives signals from the extracellular matrix, controlling cell function, differentiation and proliferation. Fibronectin is an widely studied protein which was thought to be responsible for cell adhesion to solid surfaces (Grinnell and Feld,1982). When materials are immersed in serum, 30% of the protein adsorbed is albumin, and albumin adsorption plays an important role in enhancement of cell attachment and proliferation. However, the functional mechanism of albumin adsorption on substrates and then enhancement of cell attachment is still not clear (Baszkin and Boissonnade, 1993). Howlett et al (1994) suggested vitronectin is another important protein which can promote bone-derived cell attachment to implant materials and distribution of cells. Vitronectin is required for bone-derived cell attachment, spreading and spatial distribution when exposed to substrates (Steele et al, 1992). Serum depleted of vitronectin resulted in a greatly reduced level of cell attachment, spreading and preferential spatial distribution. Protein adsorption experiments have identified vitronectin and fibronectin as the primary proteins that lead to adhesion of endothelial cells, fibroblasts and bone-derived cells. Hayman et al (1985) reported that vitronectin was the major adhesive protein in FCS

responsible for cell attachment in vitro. In competition with fibronectin, vitronectin is more available to be adsorbed onto tissue culture polystyrene (TCPS). McFarland et al (1996) demonstrated that vitronectin absorbed preferentially from serum to the amine-terminated regions of the proteins on cell membrane. Thirty minutes is enough time for bone-derived cell attachment to a quartz substrate in culture medium depleted of fibronectin, but 240 minutes are needed for bone-derived cell attachment to the same material in culture medium depleted of vitronectin. This indicates that vitronectin plays an important role for bone-derived cell attachment to quartz substrates. This dependence of cells upon vitronectin, rather than fibronectin, in serum for cell attachment is due to a failure of fibronectin to coat the substratum in the presence of the other serum protein. Vitronectin is able to coat the substratum efficiently in the presence of the other protein (Underwood and Bennett, 1989). In fresh human serum the concentration of fibronectin is about 300μg/ml and vitronectin is 200-300μg/ml. At serum concentration above 3%, deposition of fibronectin onto substratum may be inhibited by other proteins, while that of vitronectin may by unaffected (Knox, 1984). Underwood and Bennett (1989) demonstrated that under normal tissue-culture conditions with medium containing bovine serum, only vitronectin can be adsorbed onto substratum from the medium in concentrations high enough to mediate cell adhesion, spreading and growth. Fibronectin in presence of other serum proteins, shows very little adsorption onto substratum. Some cell lines can recruit fibronectin from the medium onto their surface and utilise this cell-surface bound material for cell adhesion (Rajaraman et al 1983). This may represent a way of circumventing the lack of fibronectin on the substratum.

Depending on the surface physical features, protein adsorption may be reversible or irreversible. Commonly, on hydrophobic surface, a rapidly reversible adsorption takes place and the amount of protein adsorbed may be different on different substratum. For example, albumin adsorption on HA made from cattle bones which had been immersed in BSA solution was strongly influenced by HA crystal size and the amount of protein adsorbed was changed from 0.1-0.6 $\mu g/cm^2$ (Akazawa and Kobayashi, 1996). Other research indicated that about half of the equilibrium value was reached in less than 30 sec and it took only 15 min to reach the steady state for albumin and fibrinogen adsorption on to glass (Kidane et al, 1996). In contrast, a slowly irreversible adsorption is present on hydrophilic surfaces. Lu et al (1996) suggested a diffusion-adsorption mechanism to

match protein adsorption behaviour on biomaterial surface. They used the FTIR technique to measure the adsorbed BSA protein concentration on both hydrophilic germanium crystal and hydrophobic silanated germanium crystal surfaces, and showed BSA protein diffusion from the bulk solution to the surface, followed by reversible and irreversible adsorption on the surface. The adsorption behaviour on the hydrophobic surface was extremely rapid, attaining the saturation concentration (~0.5μg/cm^2) within a few minutes, indicating the validity of the diffusion-adsorption mechanism for protein adsorption on hydrophobic surface. The adsorption on hydrophilic surface was slower and saturation was not attained even after 4 hours, and the dynamic relationship between concentration and adsorption time did not match the theoretical design. Other research found a loosely bound protein layer attached to a more securely-bound protein layer close to the material surface of hydrophilic material, which established diffusion-limited nature of the binding and decreased protein diffusivity (Vogler et al, 1995). On the other hand, Carrett et al (1996) considered that completely reversible binding for some adsorbed proteins may not exist. Some of them may be strongly bound, while others may be only loosely and reversibly bound. Carrett and coworkers suggested a model assuming that the native protein binds rapidly and reversibly, but then is slowly denatured and adsorbs tenaciously to material surface. They applied this to human serum albumin adsorption on polymer contact lenses, and suggested that, after adsorption, protein may undergo conformational changes on the contact lens surface to adapt its structure to the new environment to minimise the free energy of the surface, a situation similar to cells adhesion and space distribution on the grooved surface. Therefore in long term adsorption of human serum albumin-contact lens system, the saturation concentration was not attained until 48 h. adsorption. An equation which describes the kinetics of irreversible adsorption of protein on material was suggested by Carrett and coworkers in 1996:

$$d[DS]/dt = k_d[NS]$$

Where $[NS]$ and $[DS]$ are the number of native and denatured protein molecules on the hydrophilic surface per unit area, k_d is the rate constant for denaturation of adsorbed protein from reversible to irreversible state. The whole adsorption procedure is controlled

by denaturation rate. The amount of albumin adsorbed on to hydrophilic polymer can arrive at 2.5μg/cm$_2$ (Ishihara et al, 1991) Bone morphologenetic proteins (BMPs) are a group of very important proteins which have been used in the clinic for stimulating new bone formation at interface between implant and hard tissue(Kuboki et al, 1998). One of most interesting aspects of BMP-induced chondro- and osteo-genesis is that purified or recombinant BMPs need carriers, which are considered to be a typical drug delivery system., to induce in vivo cartilage or bone formation. In the case of biodegradable implant materials, the implant material is not only used as the carrier but also as an important cell support for migration, growth and differentiation. Sasanno et al (1993) showed that BMP can induce directly bone formation independent of cartilage formation. More recently, Saito et al (1994). showed that BMPs combined with a fibrous collagen membrane induce bone formation. The BMP-induced cell differentiation is highly dependent on the cell substratum and micro environmental conditions which are provided by BMP carriers. Recent research has related the morphology of the protein adsorption to orientation of cell growth. By artificial methods, some special grooves, 5-20μm wide, and 5-10μm high were carved onto the protein coating, trying to mimic the muscle tissue environment in vitro, so that the cells could extend their processes along the grooves and then grow along the artificial grooves (Chesmel and Black, 1995). Cellular reaction to the topography of the underlying substratum is influenced by the cytoskeletal organisation, the adhesive receptors involved and the mobility of the cells. Focal contacts might be responsible for the orientation effect on grooved substratum (Ohara and Buck, 1979). One observation was that intracellular actin fibres must be straight and not bent (Meyle et al, 1995). This implies that cells align to the substratum micromorphology to avoid a distortion of their skeleton. Another suggestion is that cells have the ability to select preferential orientation and morphology. When the cell body is conformed by the wall of the groove or other cell body, they can adjust migration direction towards the direction that needs the smallest energy. The finding of cell growth, migration along the underlying substratum will help to produce artificial organs on a designed model via tissue culture. Successful tissue culture on the grafted scaffold in three dimension may be the first step.

6.1.2 Surface modification via physicochemical methods

Histochemical observations in animal implant tests have suggested that slow solubility of calcium phosphate ceramic seems to be necessary for in vivo osteoconductivity and remodelling of the fibrous connective tissue to achieve natural bonding with implant biomaterials.

The solubility of HA, TCP, and TCP-HA ceramic biomaterials depends on the TCP content, crystal size and porosity as well pore structure (Ong et al, 1998). The dissolved ions caused a pH shift towards the acidic end. On the other hand, the dissolved components are precipitated via the interaction with the implant biomaterials. In vivo apatite, or carbonate apatite precipitated onto the implant surface, bonds the natural bone and implant together. The pH increase of immersing liquids is suggested by following reaction:

$$PO_4^{3-} + H_2O \Rightarrow HPO_4^{2-} + OH^-$$

Zeta potential changes of the immersed biomaterial also depend on Ca/P molar ratio and immersing time(Kowalchuk et al, 1995). The intensity of the negative charge changes with decreasing Ca/P molar ratio. The more the protein deposited onto the material surface, the lower the Zeta potential of the immersed material. The zeta potential changed more intensively toward negative charge in agreement with the increase in adsorption of serum protein. (Takahiro et al, 1997). Anchoring ratio and adhesion strength as well growth rate also depend on the Ca/P ratio(Ong et al, 1998). For cell anchorage, adhesion and growth, the optimal Ca/P molar ratio is around 1.60-1.64 (Suzuki et al, 1997). After immersion, bioactive glass(BG) surface was covered with an amorphous calcium phosphate-rich layer and serum protein. Quite similar to that, in an in vivo test, an amorphous calcium phosphate-rich layer was found on the BG surface which bonds with natural bone. The bonding between bone and implant BG is through a surface layer of carbonate apatite.

In this Chapter, we aimed to quantitatively investigate the serum and albumin protein adsorption onto the material surface by immersion of the calcium phosphate biomaterials in culture medium, and to evaluate the effect of protein adsorption on surface modification of both the dense and porous materials via cell culture.

6.2 Materials and methods

6.2.1 Materials

Two kinds of materials were used in this experiment and the materials were prepared as described in Chapter 2. Both dense HA-Spinel and dense HA have relative density over 93% (sample density/theoretical density) and the porosity was less than 7%. Most of the pores in the dense materials were isolated and most were smaller than 3μm. As the pores were surrounded by dense material, the immersing media were unable to enter into those pores in dense material, thus the general surface area is approximately equal to the outside surface of the disc. Highly porous HA material, had a porosity of around 45%. The mean pore size was about 315 μm, and pore size distribution ranged between 100μm to 500μm. In the case of the highly porous HA, pores were connected with each other and readily filled with immersion media. There was also porous HA materials, with porosity being around 26%. In this material, the pores were partially connected with each other and readily filled with immersion media. Both dense and porous materials were fabricated into thin discs with diameter 12 mm and thickness 3 mm. Before immersion, the discs' surfaces were polished using 1200 grill silicon carbide paper in water and sterilised in the autoclave according to the normal procedures.

6.2.2 Immersion solution and immersion procedures

Two kinds of immersion solution were used in this experiment: Ham's F-10 containing 10% v/v FCS, the composition of which was the same as that of the medium for THO cell culture, and Ham's F-10 containing 4.5% w/v bovine albumin, the concentration of albumin used was the same as that of albumin in human blood (Vander et al, 1994). Both dense and porous material discs were placed in 24 well plates, and 1.5 ml of the above immersion media was added to each well at 37°C. At time points of 1 min, 5 min, 30 min, 1 hour, 4 hours, 24 hours, and 48 hours, the discs were thoroughly washed using PBS. The absorbed protein on the disc surface was stripped using 1 ml of PBS containing 1% w/v sodium dodecyl sulfate (SDS) and the discs were sonicated for 10 min in the PBS-SDS buffer to help remove the protein from the disc surface by use of

a 6442A-Sonic Bath (DAWE Ltd., USA). Then the PBS-SDS buffer in the wells was removed into an Eppendorf container and stored at -18°C for total protein determination by the Lowry assay. Protein assay was carried out by use of the procedure as described in Chapter **3.2**.

6.2.3 The influence of immersion time and immersion media on cell growth on dense HA and dense HA-Spinel materials

Preparation of material discs:

Material discs were prepared as above and before cells were cultured on the discs, all discs had been immersed for a period of 48 hours or 96 hours in different solutions, which were:

Discs: Un-immersed,
Discs; after 48 hours immersion in Ham's F10 containing 10% v/v FCS,
Discs; after 48 hours immersion in Ham's F10 serum free,
Discs; after 48 hours immersion in sterilised distilled water,
Discs; after 96 hours immersion in Ham's F10 containing 10% v/v FCS,
Discs; after 96 hours immersion in Ham's F10 serum free, and
Discs; after 96 hours immersion in sterilised distilled water.

Cell seeding and culture:

THO cells (passage 8) were seeded on the above discs placed in 24 well plates at a density of $5x10^4/cm^2$, and 1 hour later 1.5 ml culture medium was added to each well. They were incubated at 37°C in an atmosphere of 5% CO_2 in air. The culture medium was aspirated from the wells 48 hours later and the cells on the discs were extensively washed by PBS 3 times. Each wash was for 10 min. Then the cells were digested with 1 ml 0.5M NaOH at 37°C over night(18 hours). The digested solution was removed into an Eppendorf tube and stored at -18°C for total protein determination by Lowry assay. Protein assay was carried out by use of the procedure as described in Chapter **3.2**.

6.2.4 SEM

After immersion for 4 days in culture medium, the dense HA-Spinel discs were washed with PBS 3 times (10 min) and dried at room temperature. The discs were then mounted on aluminium stubs and coated with gold in a sputtering apparatus, and finally

the specimen were examined at 15KV under a Jeol-840A Scanning Electron Microscopy in our laboratory.

6.2.5 Statistics:

All measurements were collected and expressed as means +/- standard deviations. Single factor analysis of variation [ANOVA] followed by Dunnett's test was to assess the statistical significance of results for all the experimental data. Single factor analysis of variation Student's t test was employed to assess the statistical significance of data at the same time point.

6.3 Results

6.3.1 Albumin protein adsorption

Albumin adsorption on the HA and HA-Spinel material discs is shown on Figure **6.1**. In general protein adsorption increased with increasing immersion time and was greater in porous materials. The amount of protein adsorption detected after 1 min immersion was 3.0+/-2.2μg and 4.3+/-2.7μg on dense HA-Spinel material and dense HA respectively, and 18.8+/-6.7μg and 16.6 +/-5.2μg on the 26 and 45% porous HA respectively. After 4 hours immersion the 26% and 45% porous HA material has adsorbed 48.1+/-9.4μg and 79.4+/-21.1μg protein respectively. It increased approximately 2 and 4 times up to 4 hours immersion on the 26 and 45% porous HA respectively, and appeared to stabilise thereafter suggesting that saturation of albumin adsorption on the surface of porous materials had been nearly achieved after this time. The same process was carried out on the dense materials, and protein adsorption on the dense HA-Spinel and on dense HA materials increased with immersion time. After 4 hours immersion and 24 hours immersion, dense HA-Spinel had adsorbed 12.2+/-1.4 μg and 14.5+/-3.2μg albumin protein respectively. Compared with the protein adsorbed after 1 min immersion, it increased 3 and 3.6 times after 4 and 24 hours immersion, and saturation of the dense material surface appeared to achieved after 4 hours. The dense HA, after 4 hours and 24 hours immersion, had adsorbed 17.1+/-3.1μg and 19.4+/-3.3μg respectively. Compared with the protein adsorbed after 1 min immersion, it in creased 2.9 and 3.5 times. It appeared to stabilise at the end of 4 hours immersion. Comparing the protein adsorption on dense HA to that on dense HA-Spinel discs, in general the amount of albumin adsorption on the dense HA is higher than that on HA-Spinel discs, and there is a significant difference at time points of 4 hour and 48 hour.

6.3.2 Serum protein adsorption

Figure **6.2** is the level of serum protein adsorption on the materials. In general it was similar to that of albumin adsorption onto those material surfaces, and protein adsorption increased with increasing immersion time and was greater in porous materials. The amount of protein adsorption detected after 1 min immersion 26.5+/-7.9μg and

19.8+/-8.1 μg on HA with 26% and 45% porosity respectively. Four hours later, adsorption was 47.8+/-4.4μg and 102+/-32.6 μg on HA with 26% and 45% porosity respectively. It increased approximately 1 and 4 times up to 4 hours immersion on HA with 26% and 45% porosity respectively, and adsorption appeared to stabilise thereafter suggesting that saturation of protein adsorption on the surface of porous materials had been achieved after 4 hours immersion. For the dense HA-Spinel materials, there were 3.0+/-2.2μg and 13.5+/-3.2μg protein adsorbed after 1 min and 24 hours immersion respectively and it appeared to stabilise thereafter. With the dense HA, there were 5.4+/-0.7μg and 22.8+/-2.9μg protein adsorbed after 1min and 24 hours immersion respectively and adsorption appeared to stabilise thereafter suggesting saturation of the material surface after 24 hours immersion. Similar to albumin adsorption on dense materials, the amount of serum protein adsorption on dense HA was higher than that on HA-Spinel, and there was a significant difference at time points of 4 hour and 24 hour. To facilitate comparison of the different protein adsorption on the same material, the data have been presented on the different materials. Figure **6.3-6.4** is the illustration of the results. Although some significant differences between the adsorption of albumin and serum proteins was detected on HA of 45% porosity, in general, both proteins were adsorbed to similar extents on the materials.

6.3.3 Effects of immersion media and immersion time on THO cells growth

Figure **6.5** shows the effects of different immersion solutions and different immersion times on THO cell growth on dense HA-Spinel and dense HA discs. For the growth of THO cells on the dense HA-Spinel discs, there was no significant difference between different immersion solutions, and there was no significant difference between immersion times. With THO cell growth on immersed dense HA material discs, apart from the discs that were immersed in the medium FCS free for 96 hours, there was no significant difference between different immersion solutions and there was no significant difference between different immersion times. In terms of total protein of the cells, there was a significant difference between samples which were immersed in the serum free medium for 48 hours and samples which were immersed in the serum free medium for 96 hours; the former was 46.3+/-5.1μg protein per sample and latter was 24.4+/-7.8μg protein per sample. There are two facts which should be noticed: after immersion for 96

hours, the growth rate of the cells, in general, was lower than that of the cells cultured on the discs that had been immersed for 48 hours. In addition, the total protein on un-immersed dense HA-Spinel samples and on un-immersed dense HA sample was higher than the total protein on the discs which had been immersed in the culture medium serum free. Total protein values on the un-immersed dense HA-Spinel and dense HA were 31.1+/-11.0μg and 37.3+/-4.3μg respectively. Total protein valves on the dense HA-Spinel and dense HA which were immersed in serum free medium for 96 hours were 28.5+/-6.6μg and 24.5+/-7.8μg. There was a significant difference between total protein on un-immersed dense HA and total protein on HA which was immersed in medium FCS free for 96 hours.

6.3.4 SEM observation

After being immersed for 96 hours and observed by SEM, a lot of white tiny particles with size ranging from 0.1-1 μm were found on the dense HA-Spinel surface. The distribution and morphology of these deposited tiny particles is shown on Photograph **4.5.**

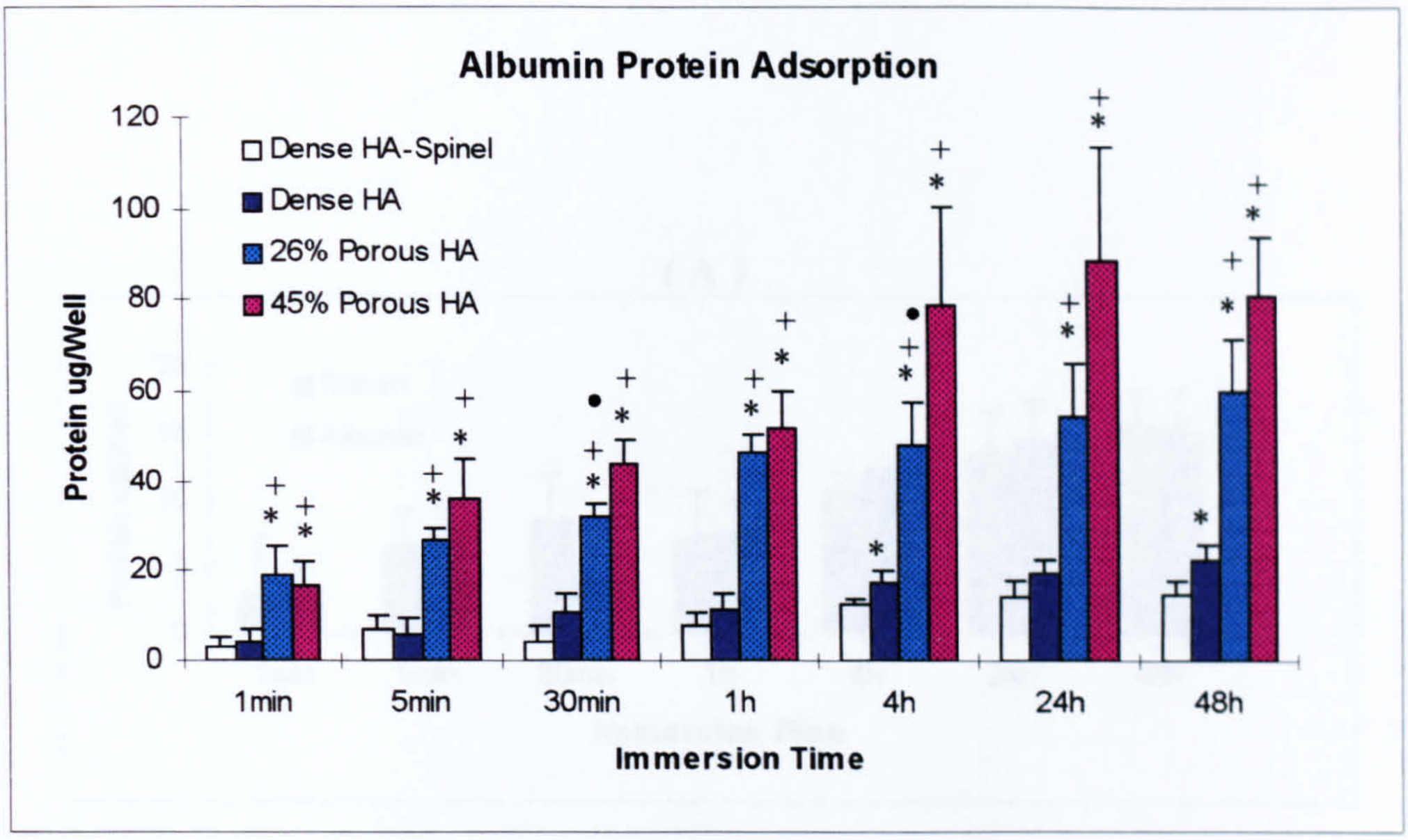


Figure 6.1. Bar chart showing albumin protein adsorption on dense HA-Spinel and HA biomaterials. The adsorption was saturated at the time point of 4 hours. Results are mean +/-SD, n=4. *P<0.05, by ANOVA followed by Dunnett's test, compared with dense HA at same time point. $^{+}$P<0.05, by ANOVA followed by Dunnett's test, compared with dense HA-Spinel at same time point. $^{\bullet}$P<0.05. compared with porous HA with 26% porosity at same time point, by unpaired Student's t test.

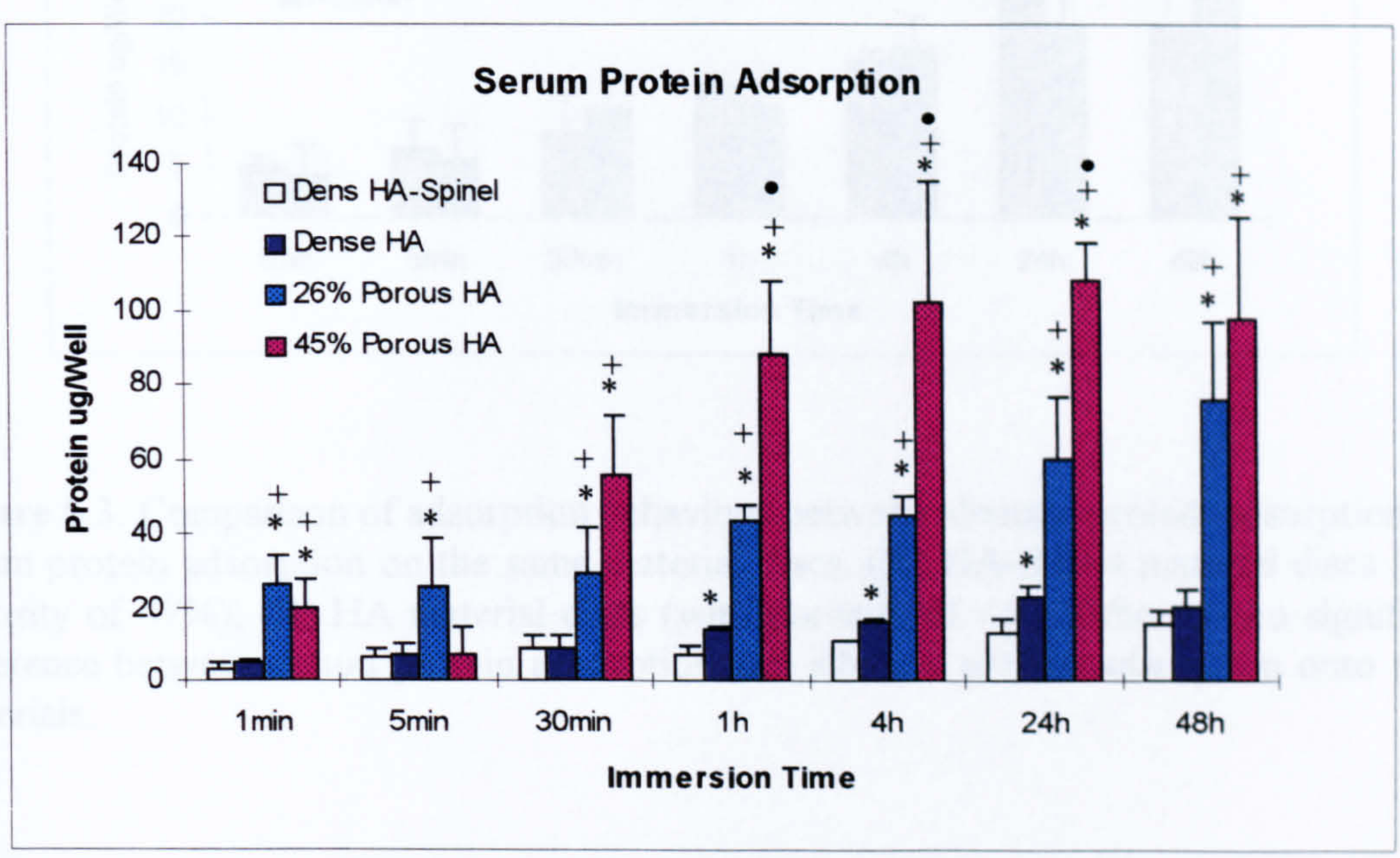
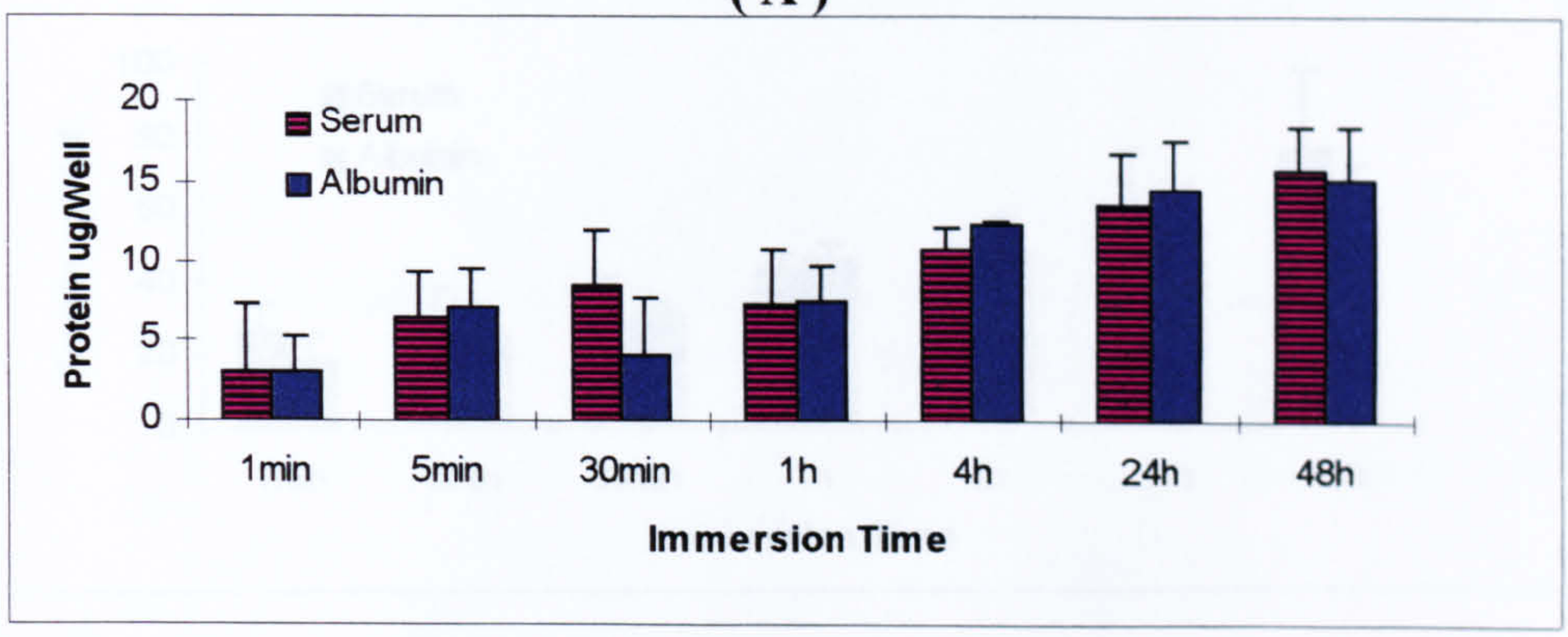


Figure 6.2. Bar chart showing serum protein adsorption on dense HA-Spinel and HA biomaterials. The adsorption was saturated at the time points between 1 hour and 4 hours. Results are mean +/- SD, n=4. *P<0.05, by ANOVA followed by Dunnett's test, compared with dense HA at same time point. $^{+}$P<0.05, by ANOVA followed by Dunnett's test, compared with dense HA-Spinel at same time point. $^{\bullet}$P<0.05. compared with porous HA with 26% porosity at same time point, by unpaired Student's t test.



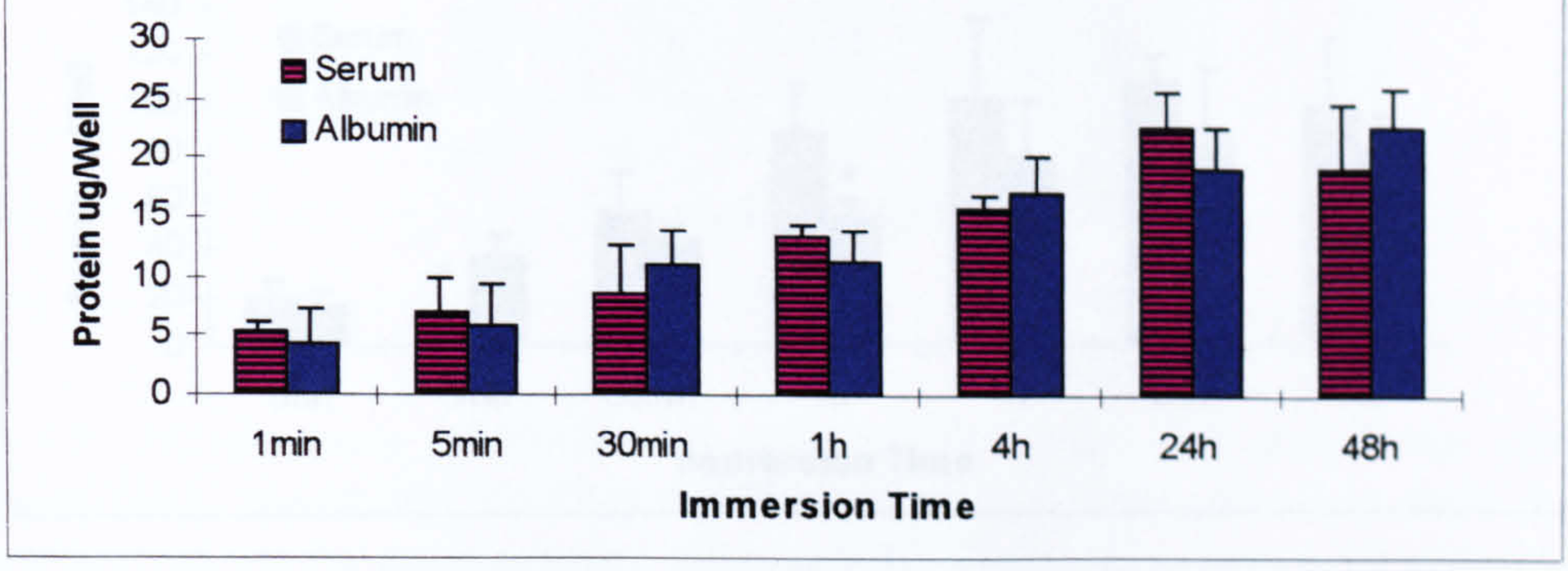
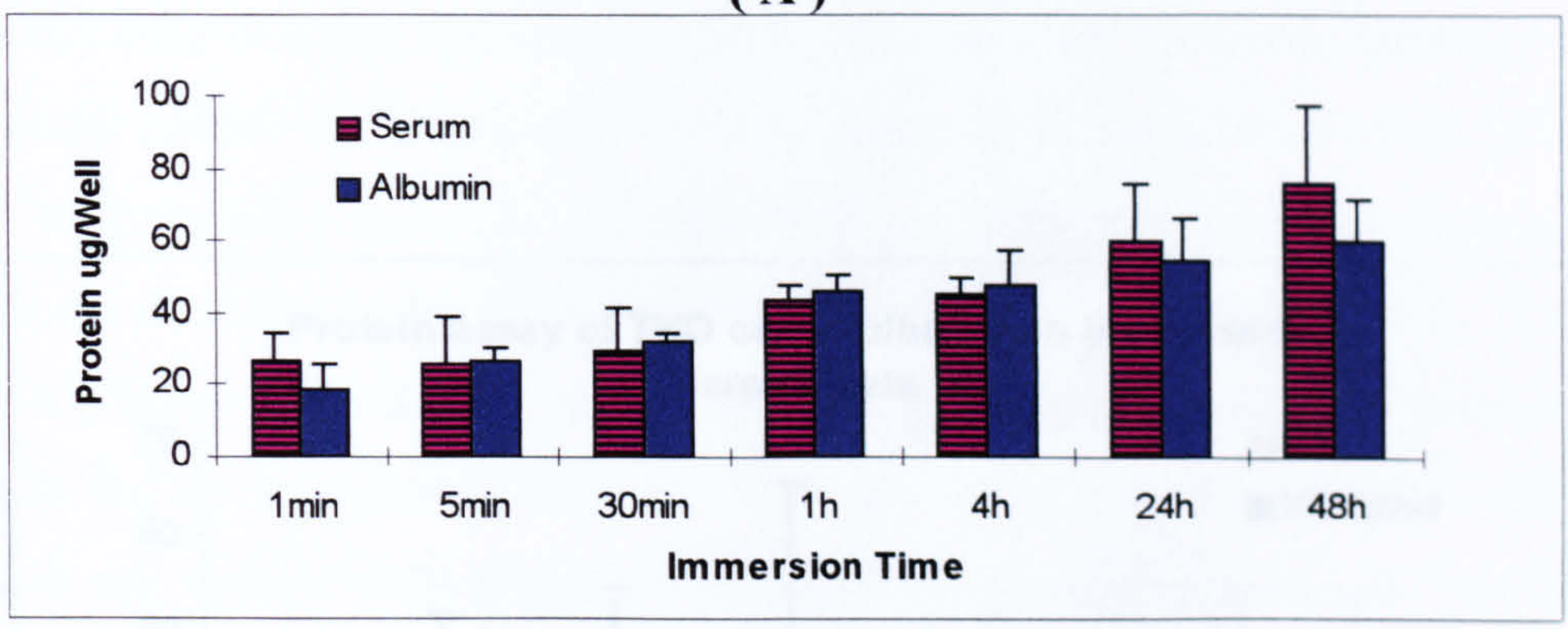


Figure 6.3. Comparison of adsorption behaviour between albumin protein adsorption and serum protein adsorption on the same material discs. (A) HA-Spinel material discs (with porosity of <7%); (B) HA material discs (with porosity of <5%); there is no significant difference between serum protein adsorption and albumin protein adsorption onto these materials.

(A)



(B)

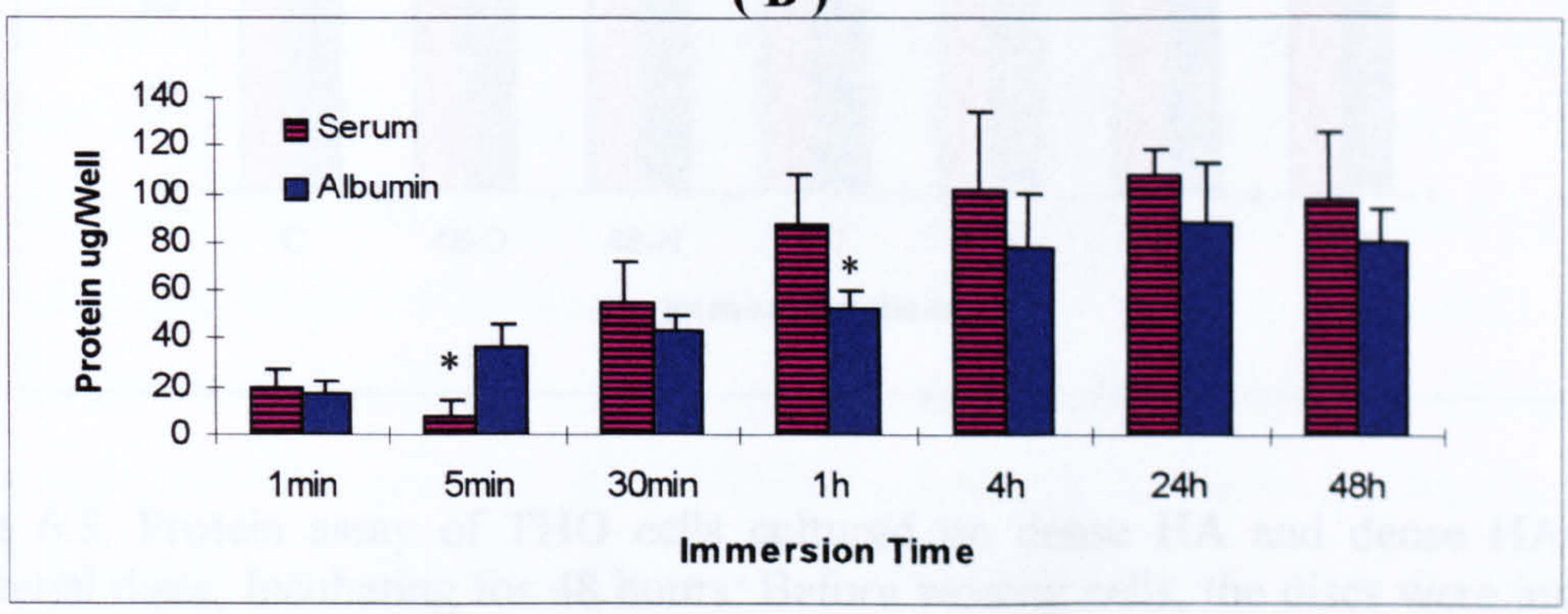


Figure 6.4. Comparison of adsorption behaviour between albumin protein adsorption and serum protein adsorption on the same material discs. (A) HA material discs (with porosity of 26%); and (B) HA material discs (with porosity of 45%). *P<0.05, data compared with serum protein at same time point, by unpaired Student's t test.

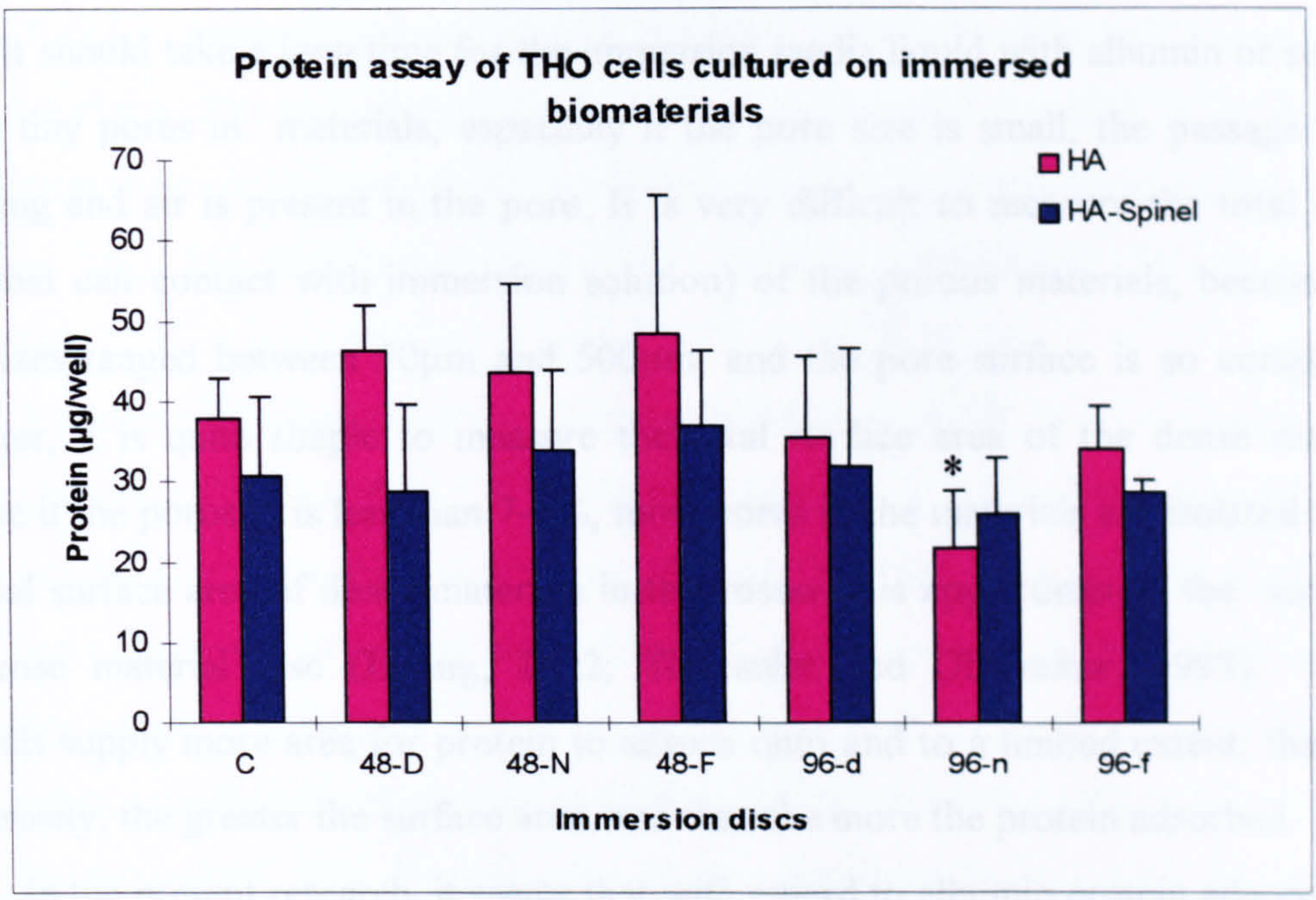


Figure 6.5. Protein assay of THO cells cultured on dense HA and dense HA-SPinel biomaterial discs, Incubating for 48 hours. Before seeding cells, the discs were immersed in different solutions for 48 hours and 96 hours. *P<0.05, was compared with 48-N, and un-immersed discs C by use of ANOVA followed by Dunnett's, n=4. C: Dense un-immersed HA-Spinel or HA; 48-D: Immersed in Distilled water, 48 hours; 48-N: Immersed in the medium FCS free, 48 hours; 48-F: Immersed in the mediun with FCS, 48 hours; 96-d: Immersed in distilled water, 96 hours; 96-n: Immersed in the medium FCS free, 96 hours; 96-f: Immersed in the medium with FCS, 96 hours.

6.4 Discussion

In the present research, a greater albumin adsorption on porous HA and highly porous HA than on dense HA-Spinel material was expected. However, basically we must consider the specific surface areas of the bulk materials, as the surfaces of the pores in the interior of the porous materials will contribute, and the pores are connected with each other. It should take a long time for the immersion media liquid with albumin or serum to fill the tiny pores in materials, especially if the pore size is small, the passage way is zigzaging and air is present in the pore. It is very difficult to measure the total surface area (that can contact with immersion solution) of the porous materials, because most pore sizes ranged between 50μm and 500μm, and the pore surface is so complicated. However, it is quite simple to measure the total surface area of the dense materials, because if the porosity is less than 7-9%, most pores in the materials are isolated and, so the total surface area of dense materials in this research is approximately the surface of the dense material disc (Huang, 1992; Thummler and Oberacker, 1993). Porous materials supply more area for protein to adsorb onto and to a limited extent, the higher the porosity, the greater the surface area, and then the more the protein adsorbed.

In the present research, it seems that with regard to albumin protein adsorption on the HA discs with 45% porosity, the adsorption was nearly saturated at a time point of 4 hours, while with regard to serum protein, the adsorption was saturated at a time point between 1 and 4 hours. For the HA material with 45% porosity, approximately 20% of the amount of albumin protein adsorbed after 48 hours (81.2+/-13.3μg/well) has been adsorbed within 1 minute (16.6+/-5.26μg/well). For the HA material with 26% porosity, approximately 30% of the amount of albumin protein adsorbed after 48 hours (60.2+/-11.4μg/well) has been adsorbed within 1 minute(19.0+/-6.7μg/well). Similarly, with regard to serum protein adsorption on to porous HA materials, for the HA material with 45% porosity, approximately 20% of the amount of serum protein adsorbed after 48 hours (97.6+/-27.8μg) has been adsorbed within 1 minute (18.7+/-8.1μg/well). For the HA material with 26% porosity approximately 30% of the amount of serum protein adsorbed after 48 hours (75.7+/-21.2μg/well) has been adsorbed within 1 minute(26.0+/-13.5μg/well). Those results imply that prolonged immersion may not be required in order

to enhance cell adhesion to the materials, because we found that at least 10 min were required to initiate cell attachment onto the material surface during the seeding process. The adsorption process was basically the same for both albumin adsorption and serum protein adsorption, though the amounts of proteins adsorbed were different with porous HA materials. For dense HA-Spinel materials, at the end of 1 minute immersion, there was less than 3.0μg/well albumin protein adsorbed onto the material surface, but at the end of 4 hours immersion, more than13.2μg/well albumin protein had adsorbed onto material surface. This represents over the half of the proteins adsorbed at the end of 48 hours immersion . At the end of 48 hours immersion, albumin protein and serum protein adsorbed onto HA-Spinel material were 16.0+/-3.2μg/well and 17.4+/-2.6μg/well respectively.

It seems that it has to take more time for dense materials to achieve saturation of protein adsorption. The reason for this is not clear and it is suggested that in fact the dense material disc is not absolutely dense, and that there are some very tiny pores within the material connected to the material surface, even though they are not connected with each other. As air is present in the tiny pores, it will impair entry of immersion liquid into the tiny pore, therefore it will require more time for immersion liquid to substitute for the air in the tiny pores. For example, the results show that it may take 24 hours for saturation of serum protein adsorption on dense HA material discs. Compared with the tiny pores in the dense material, the rather big pore diameter and inter-connection of pores with each other in the porous materials, means that the air in the pores is easier to displace, and subsequently, less time is required for protein adsorption onto the surface of the porous materials. Though tiny pores were also present in the porous material, the amount of protein adsorbed in these is within the error limitation. The curves in Figure **6.1-6.2** demonstrated that when the material density was the same, though the amounts of the protein adsorbed at some time points are different, the general trends are the same. That is with increasing immersion time, the amount of protein adsorbed onto materials surface increased, and at a time point, adsorption would be saturated. Figure **6.3-6.4** displayed that there was no great difference between serum and albumin adsorption on the materials when the composition and porosity were same. In other words, if the composition and porosity of the material are the same, they have the same adsorbing rate

for serum and albumin proteins under the present conditions of serum concentration and albumin concentration. The reason may be that albumin was also present in the serum solution, therefore, the same adsorbing mechanism can be present in serum protein adsorption and albumin adsorption in this research.

Depending on the interaction between immersion materials and proteins, as well between the immersion material and solution, adsorption may be classified as physical adsorption (van der Vaals force), chemical adsorption, and biochemical adsorption. Physical adsorption may be a completely reversible process. In chemical adsorption, chemical reactions between immersed material and the solution take place, for example, in terms of Ca^{2+} and PO_4^{-3} dissolution and precipitation, c-HA crystal formation on the material surface and in the immersion solution usually helps to perform chemical adsorption. Proteins in solution also may mediate the chemical adsorption. Radin et al (1998) demonstrated that c-HA precipitation on immersed calcium phosphate materials was observed after 3-week culture with osteoblasts. No significant surface changes were detected after immersion in an acellular serum containing solution. In contrast, after immersion in an acellular serum free solution simulating the ionic composition of plasma, c-HA was abundantly formed on the immersed material. Radin also found that the addition of serum protein further delayed the reaction on HA, TCP etc. Another researcher (Blumenthal, 1989) also suggested that the reactions leading to c-HA formation on the surfaces of ceramic materials were impeded by the presence of protein in the medium. Serum albumin and osteocalcin are known as crystallisation inhibitors in bone. Martin and Brown (1994) also suggested that proteins inhibit HA formation and their results show that when decacine HA ($Ca_9(HPO)(PO_4)_5(OH)$ was formed by reaction of the particulate ceramics as follows:

$$\mathbf{6CaHPO_4+3Ca_4(PO_4)_2O \Rightarrow 2Ca_9(HPO_4)(PO_4)_5(OH) + H_2O}$$

the chemical reaction was finished in DI water in 7 hours. But 72 hours was required if the above reaction took place in the presence of serum. When material was immersed in medium with serum, albumin rapidly coated on HA surface and formed a protein layer. However, the mechanism of protein interference with c-HA formation is still unclear. The strong adsorption of protein on the material must impair the interflow of carbon, Ca^{2+},

PO_4^{-3}, and OH^{-1} between material and solution, and consequently impair formation of c-HA crystals (Muster, 1992). Biochemical adsorption between protein and material surface is a very complicated process. Protein adsorption on the material surface mainly involves biochemical adsorption, but also involves the other two kinds of adsorption. Biochemical adsorption of protein can be realised via receptor-ligand combination. One possible method is protein adsorption onto material via the combination of the receptor site of protein membrane and OH group in HA materials. However, it is difficult to understand that protein also can be strongly adsorbed onto inorganic materials without an OH group, such as TCP. Blumenthal (1989) suggested that molecules with both P-O-P and P-C-P bonds strongly adsorb to the surface of calcium phosphates, with several consequences. Akazawa and Kobayashi (1996) reported that the ability of HA as an adsorbent is dominated by the pore structure and by the physiochemical nature of solid surfaces e.g. crystal size, surface tension, and wetting angle. Their research results show that the adsorption characteristics of BSA on HA obeyed the Langmuir equation. In my research, we also found that after immersion for 4 hours, the amount of proteins adsorbed onto rather dense discs (porosity: 26%) still increased, I suggested that some tiny pores were present in the rather dense materials and it would take longer time to substitute air for liquid in the pores. I tried to use the SEM in our laboratory to investigate the adsorption of protein on dense HA-Spinel material. Many tiny white particles have been found on the immersed samples, but we still did not recognise what kind of molecules they were. However, the SEM observation of the tiny particles (Shown in Photograph **4.2)**, the particle size and the particle distribution were similar that of fibrinogen deposited on poly-butyl methacrylate (poly BMA) after it was immersed in human plasma for 60 min at 37°C (Ishihara et al, 1991).

According to the data in figure **6.5**, in terms of total protein determination, except for the dense HA discs immersed in the FCS free medium, no notable differences were found in the growth rate of THO cells between the immersed material and the un-immersed material. The general trend was that immersion for a long time was not necessary for cell attachment and proliferation, because protein adsorption was saturated in 4 hour on dense HA-Spinel, dense HA, and porous HA biomaterials.

Based on the results of the present research, it is suggested that in order to accurately evaluate the effects of immersion media and immersion time on protein

adsorption and cell proliferation as well as differentiation, a longer period of cell culture may be required, and, for c-HA formation, a longer period of immersion may be required. Anselme et al (1997) showed that during immersion of HA in the culture medium DMEM, a high release of calcium or phosphate ions was observed between 7 and 15 days. Human bone-derived cells grew only on samples previously immersed for 15 or 22 days. Anseme et al (1997) reported that the rate of Ca and P dissolution in DMEM reached a maximum after 7 days and a stabilization after 4 weeks of immersion. Magnesium has a considerable inhibitory effect on the formation and growth of HA crystal (Tenhuisen and Brown, 1997). Magnesium interferes with HA formation by poisoning nucleation and causing $(Ca, Mg)_3(PO_4)_2$ to form (Martin and Brown, 1994). In my research even if the HA-Spinel material was immersed in cell culture medium for 21 days, no HA crystal was observed on the surface (See Chapter **5.3**). Based on the mechanism that newly forming bone between implant and tissue is mediated by c-HA deposition on the implant materials, it will need more time for HA-Spinel material to conduct the osteointegration processes. In the present research, although the amount of adsorbed protein on HA materials was higher than that on HA-Spinel, and there was a statistically significance at several time points, it is difficult to judge how much interference with protein adsorption is caused by Al and Mg ions or Al-Mg-O compounds. However, we considered that immersion for a long term is not required because over one third of the protein adsorbed at saturation will adsorb onto the material surface in a few minutes. We did not identify what the tiny white particles were but we suggest that the tiny white particles were a kind of protein crystals. It should be determined by FTIR technique or immunochemistry methods. This will be the subject of further research.

Chapter 7 General discussion and conclusions

The aim of the final chapter is to present an overall summary of my main research. Looking back at the research during the past three years, in general, it may be described as "Processing, characterization biocompatibility evaluation of calcium phosphate biomaterials in vitro." However, in my opinion, there is a lot of further work we could have dealt with, if I had more time. It should be said that the present research spreads over rather a wide inter- disciplinary approach. This is associated with my background of materials science and materials research coupled with clinical experience of surgeons for many years. Dense and porous calcium phosphate biomaterials which are aimed at two different applications in the clinic were prepared. Dense calcium phosphate is used to bridge broken or damaged bone originating from various traumas. The porous calcium phosphate is use to fill vacancies in bone where usually tumors have been cut away. Biological effects of biomaterials are directly related to composition and structure of the materials, and practical application is associated with the biomechanics. In order to improve the mechanical properties, $MgAl_2O_4$ was added into the calcium phosphate. We hoped this would not impair the special biological performance of the calcium phosphate. The problems I faced after adding Spinel were: What is the microstructure? Is there any intermediate phase present in the HA-Spinel composite? What happens to the mechanical properties? What happens to the biological character?

Although both above dense and porous biomaterials have been successfully applied in the clinic, some important aspects of the tissue response to the implants remain unknown. Therefore, in the present research, I prepared dense and porous materials, tested their mechanical properties, and investigated microstructure, phase structure and phase transformation. The processing method of ceramic biomaterials described in Chapter **2** is the method applied worldwide. In clinical application, the function of porous biomaterials when filling bone vacancies, is to supply a scaffold for tissue anchoring and to encourage new bone formation, by biodegrading and being gradually substituted by newly formed bone. As porous biomaterials have a specially important role, highly porous calcium phosphate has also been fabricated and tested in the present research. X-ray spectrum analysis has shown that after being sintered at 1250°C, for 2-4 hours, there was

no phase transformation in the materials originated from HA powder. OH groups remained in the crystal and there was no obvious *HA-TCP transformation. SEM* observation has shown that the pores connected with each other in the porous material and the pore size was distributed between 50-500μm which is close to that of human cancellous bone, the latter being 50-400μm (Seeley et al, 1995). X-ray spectroscopy has shown that TCP and Spinel phases were present in sintered HA-Spinel dense materials. It means that a phase transformation of HA-TCP had taken place in the dense material during sintering at elevated temperature (1450°C, 2-4 hours). I have not found TCP-Spinel compounds in the sintered material. SEM observation has shown that sintering at the temperature close to the melting temperature of TCP and/or HA did not produce full density materials, and some pores with the diameter smaller than 3 μm still were left in the sintered materials. They were not connected with each other.

In order to use biomaterials in the human body, it is necessary to make toxicity evaluations. Four kinds of biological assay have been performed in the present research to evaluate cytotoxicity of the biomaterials: Intracellular GSH content, intracellular MTT reduction, Lowry assay for protein content, and the ability of cell to synthesise collagen. As the biomaterials prepared in this research were inorganic biomaterials and did not degrade significantly in short term, the interflow between the materials and environment was limited to the material surface. Based on this consideration, I made extracts of the materials two times to investigate the action of culture medium on material surface. Cytotoxicity evaluation demonstrated that compared with the negative control, GSH content, MTT reduction, and total protein were significantly decreased in the first extract of HA-Spinel material. In contrast with that, GSH content, MTT reduction, and total protein were not significantly different in the second extract of HA-Spinel material. The data show that slight cytotoxicity was presented in the first extract and that after extraction, or immersion, in other words, this slight cytotoxicity could be eliminated. These results suggest that before the materials are to be applied in surgeries, suitable treatment by immersion and/or extraction is necessary to reduce the reaction of materials with surrounding tissue. It should be considered that the ALP assay is the one of the most sensitive indices of cytotoxicity in osteoblasts. In the present research, ALP assay was subject to interference from the materials and could not be used. Of the above four

indirect cytotoxicity indices MTT is most insensitive, and depends on several factors like seeding density, incubation time as well as cell type. The data read on the micro culture plate are directly controlled by cell density in the wells. In my opinion, GSH content and Lowry assay for total protein content are sensitive indices of cytotoxicity and are the ideal methods to evaluate material's toxicity in vitro. GSH is more sensitive than the Lowry assay but Lowry assay can give the more reliable data. Although only slight toxicity is present in the material, both GSH and Lowry assay can tell the difference between the first extract and second extract. Collagen synthesis is the most important function of osteoblast cells. Collagen synthesis was impaired in the first extract, however, this is less marked, so that compared with the GSH and Lowry assay, collagen synthesis is not a sensitive index of cytotoxicity. Expression of collagen synthesis usually needs rather a long time of cell incubation, and perhaps I should have incubated the cells for longer. When the toxicity of the materials is not enough to impair cell growth and proliferation, it is very important to check that a suitable cell density and incubation time have been used. The faster growth rate of FFC cells resulted in the action of the extracts on FFC cells being more marked than on THO cells.

The research show that both THO and FFC cells can attach onto and grow on both dense HA-Spinel and porous HA biomaterials. This was shown in Chapter **3** by the relationship between cell proliferation and culture time. Normal morphological expression of both THO and FFC cells are displayed on both dense HA-Spinel and porous HA materials. Extracellular collagen fibre synthesis on the biomaterials was observed and the density of collagen fibers increased with culture time. This displayed that human osteoblast cells have normal secretion function on the biomaterial, and demonstrated indirectly the bone forming ability of human osteoblast cells on the dense HA-Spinel material. It is difficult to judge which cell line, FFC and THO, is more suitable to be used to evaluate the cytotoxicity of the materials, particularly as a more sensitive method such as ALP assay could not be applied. Both THO and FFC cells are available to be applied for testing toxicity of the implant materials in vitro.

These research results shows that both dense and porous calcium phosphate biomaterials possess strong ability to adsorb serum and albumin protein. The general tendency is the amount of protein adsorbed onto material surfaces increased with time and achieved saturation in 2-4 hours. 20-30 percent of the saturated protein level

adsorbed onto material surface in several minutes. It means that before cells attach to material surface, proteins in culture medium have adhered to material surface and cells then adhere to material surface mediated by the proteins which have adsorbed to its surface. If preferential adsorption of proteins occurred on some parts of the material surface, then attachment of cells onto the material surface also will be selective. Spinel incorporation in the material may limit protein adsorption. The Al and/or Mg ions in the materials or in the medium dissolved from the materials may impair protein adsorption onto the material surface, although the mechanism of the limitation is not clear. HA, TCP, and other calcium phosphate biomaterials may have a special mechanism for proteins adsorption. Kokamoto et al (1998) demonstrated that RGD peptides regulated the specific adhesion of osteoblasts to calcium phosphate bioceramics but not to titanium.

In general, though this research has systematically evaluated the biocompatibility of calcium phosphate biomaterials, especially the cytotoxicity of the biomaterial via in vitro model, it still lacks some important evaluations. Firstly, the ALP assay should be carried out as ALP displays high activity in osteoblasts, reflecting both differentiation and maturation (Anderson 1989; Macnair 1996). Many researchers have reported that ALP assay is the most important index of osteoblast function. Secondly, if I had time, I would have prepared a series of HA-Spinel materials with different percentages of Spinel in the materials and compared the effect of composition on the biological characteristics of the materials in vitro. In the present research, both dense HA-Spinel and porous HA were fabricated in accordance with the requirements of the clinical application. Also I would fabricate the materials with different percentages of Spinel and different porosity. Finally, in my research, only one cell type was involved to evaluate compatibility of the implant materials. I suggest that for the bone substitutes, apart from the action of implant materials on the surrounding tissue, many factors will react to implant materials. Most important is the reactions of osteoclasts and osteoblast simultaneously. Osteoclasts resorb implant material and osteoblasts form new bone on the implant materials at same time. In vitro, usually, only one cell line is cultured to investigate material characteristics. The result in this may be quite different from the situation in the clinic. To increase clinical relevance, a co-culture model of osteoblasts-osteoclasts would be worthy of investigation. Co-culture of osteoblasts and osteoclasts may be closer to the clinical situation. The research system may be very complicated in a co-culture model, but the

interaction between material and cells as well as regulation between osteoblasts and osteoclasts would be represented. If I had the opportunity, the co-culture of osteoblasts and osteoclasts should be my first consideration. To do this, stromal stem cells are proposed which divide and regenerate themselves and produce committed bone cell progenitors. The progenitor cells can divide further and result in the production of osteoblast cells. The progenitor population is not self renewing and the osteoblast population is terminally differentiated. A hypothetical thought is that osteoclasts also derive from bone marrow. Bone marrow stem cells may be differentiated into osteoclast progenitors, osteoclast precursors, and osteoclasts (Burger and Nijweide, 1991). Therefore, both of osteoclasts and osteoblasts could be simultaneously differentiated from marrow cells by the development of their own stem cells respectively. Stem cells are capable of proliferation but remain undifferentiated until the correct inducing conditions are applied, whereupon some or all of the cells mature to differentiated cells. Many factors would effect cell differentiation and proliferation during co-culture. It may be useful to think of a cell culture as being in equilibrium between stem cells, undifferentiated but committed precursor cells, or mature differentiated cells, and that equilibrium may shift according to the environmental conditions. Routine serial passage at relatively low cell densities would promote cell proliferation and little differentiation, while high cell density, low serum and appropriate hormones would promote differentiation and inhibit cell proliferation (Freshney, 1993). However, at present bone osteoclast-like cells are mainly derived from marrow stem cells but osteoblasts are mainly isolated from animal or human bone directly by enzymatic digestion (Zallone and Teti, 1991).

It is very clear from this research that cytotoxicity testing techniques used in the present research can give a good indication of the toxic effects of materials extracts. The tests were sensitive even to a slightly toxic chemicals. However, the toxicity of chemical can only be evaluated relative to the control materials. Other problems are that cell culture techniques can be prone to bacterial, viral and fungal contamination, and a large number of samples are needed to give representative and reproducible results. Cell culture techniques and environments do not completely replace biocompatibility evaluation of biomaterials in animal model. For example, tissues can grow into porous materials in every direction in an in vivo model, but cells grow into porous materials only

in one direction in vitro as the effect of gravity on cells is unable to be negative in an in vitro environment. It should be noticed that on considering the possibility of cell migration through the porous sample via lateral pores, the porous materials with big, connected pores are not suitable to culture cells on quantitatively. Cell migration out from the pores during culture may result in mistakes. This situation does not mimic that in vivo where cells can migrate into a porous scaffold in every direction. Therefore, how to mimic the cells or tissue growth on the materials via in vitro model is still a problem to be dealt with.

APPPENDICES

I Buffer and Salt Solutions

Tris Buffer Saline (TBS)

To 450ml distilled water the following compounds were added:

NaCl	4.00g
Na_2HPO_4	0.05g
Glucose	0.5g
Tris	1.5g
Phenol red 0.5% (w/v)	1.5ml

The pH of the solution was adjusted at room temperature to 7.7 with 1M HCl. The volume was made up to 500ml with distilled water and autoclaved at 15lbs for 15min. Its sterility was checked for three days with Brain Heart Infusion (BHI) and Sabouraud's medium (SAB).

Versene in PBS, pH 7.2

The following compounds were dissolved in 1500ml distilled water. Its pH checked to see if it was 7.2, and it was then autoclaved at 15lbs for 15min. The sterility was checked for three days with BHI and SAB.

NaCl	12.00g
KCl	0.30g
Na_2HPO_4 anhydrous	1.73g
KH_2PO_4	0.30g
EDTA	0.30g
Phenol red 1% (w/v)	2.25ml

Phosphate Buffer Saline (PBS)

To prepare 400ml of PBS bufer, 2 PBS tablets were dissolved in 400ml distilled water. This gives a solution of 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride. At 25°C, the pH is 7.4. This solution was stored at 0-5°C.

Composition of Hank's II Buffer

To prepare 500ml of Hank's II buffer, the following were dissolved in 450 ml distilled water. The pH was adjusted to 7.4 using 5M NaOH.

$NaHCO_3$	1.05g
Hepes	1.50g
$CaCl_2 \bullet 2H_2O$	147mg
Hanks Stock (x10)	50ml

PBS/BSA/Azide Buffer

This buffer is used in immunochemical staining to dilute the FITC labelled antibody so only small quantities were made up at any time. The composition is shown as below:

PBS	50ml
BSA	5g
5% Na Azide	1ml

Mounting Medium

This is used to mount cover slips onto materials after immunofluorescent antibodies have been applied. The composition is shown as below:

PBS/BSA/Azide buffer	8ml
Glycerol	2ml
O-Phenylenediamine	200mg

It consisted of PBS/BSA/Azide, 20% (v/v) Glycerol and 20 mg/ml O-phenylenediamine. Stored at 4°C in the dark for 3 days. Turns dark yellow when it goes off.

II Stock Solutions

Penicillin Streptomycin

Penicillin	6g/l in distilled H_2O
Streptomycin	10g/ in distilled H_2O

L-Glutamine (x100)

Dessolve 30mg/ml in distilled H_2O

L-Ascorbic acid (50mg/L)

Dissolve 25mg L-Ascorbic acid into 5ml Ham's F-10 medium.

Add this to 495ml Ham's F-10 to give 50 mg/l.

Sodium Pyruvate (100mM)

Dissolve 1.1g in 100 ml distilled H_2O

Collagenase (0.5 mg/ml)

Dissolve 50mg of collagenase type IV in 100ml Hanks II buffer.

All stock sollutions were sterile filtered with 0.22μm fillter units and aliquoted before being stored at -20°C.

III Cell Culture Media

Dulbecco's Modified Eagles Medium (DMEM)

Autoclaved distilled H_2O	450ml
$NaHCO_3$ (7.5% w/v)	25ml
Dulbecco's medium 10x concentration	50ml
FCS	50ml
L-Glutamine	5ml stock solution
Na Pyruvate	5ml stock solution
Penicillin Streptomycin (PEST)	5ml stock solution

Ham's F-10 Medium

Ham's F-10 medium	450ml
FCS	50ml
L-Ascorbic acid	5ml stock solution
PEST	5ml stock solution

Both DMEM and Ham's F-10 was checked for sterility with SAB and BHI for three days before use. Stored between 0-5°C.

Freezing Media

FCS	8ml
Culture medium	10ml
DMSO	2ml

This was used when stocks of cells were to be frozen in liquid nitrogen.

IV Basic Culture Techniques

All culturing and experiments with cells were performed in class II flow cabinet (Envair), the surface of which was swabbed with 70% (w/w) ethanol solution before and after use.

Freezing Stocks of Cells

An even suspension of cells in the appropriate medium was prepared. The cells were counted and sedimented by centrifugation in a sterile universal container (conical bottom) at 500g for 5min. The supernatant was poured off and the cell pellet resuspended in ice-cold freezing medium at approximately $5x10^6$ cells/ml using a sterile plastic pipette. 1ml quantities of the suspended cells were then placed in sterile cryogenic tubes and placed in a -70°C freezer overnight. After this period, the tubes were transferred to liquid nitrogen storage.

Thawing and Reviving Stocks of Cells

The cells to be revived were retrieved from liquid nitrogen storage and brought to 37°C by holding the cryotube in a water bath until the cells had thawed. These cells were immediately transferred to a flask containing 10ml growth medium, diluting the DMSO present in the freezing medium. The cells were allowed to adhere to the surface overnight before the medium was replaced with fresh growth medium.

Checks for Bacterial and Fungal Growth

To check for bacterial and fungal growth, 500ml of BHI and SAB were made up according to the manufacturers instructions and autoclaved. 20ml aliquoted BHI and SAB were stored at room temperature until required. When a liquid was to be tested, 2-3ml of the liquid was poured into SAB and BHI solutions and left at 37° for up to 1 week. If the solution remained clear, there was no infection. BHI tests were for bacterial growth and SAB for fungal growth.

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