# USE OF HUMAN AUTOLOGOUS CHONDROCYTES & MESENCHYMAL PROGENITOR CELLS IN CARTILAGE REPAIR TECHNIQUES

*By*

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## Abstract

Due to articular cartilage's limited intrinsic repair potential, the treatment of cartilage damage in the younger patient provides a major challenge.

The relative failure of many synthetic solutions, has led to the growing interest in the development of cell-based repair systems. In general two strategies have been considered. The first approach is to enhance the intrinsic healing capacity of both the cartilage and subchondral bone through the release of mesenchymal progenitor cells, however, to date, these techniques have only led to fibrocartilaginous repairs, which lack long term durability. An alternative approach involves the use of tissue engineering strategies to elicit a biological repair. The most commonly used practice in the clinical setting is Autologous Chondrocyte Implantation (ACI).

This thesis presents a review of the literature in the subject of cartilage repair, looks at the cell sources available to develop repair systems and investigates factors that might influence these cells. In this study, an *in vitro* system was used to promote the chondrogenic potential of these cells allowing for study of some of the factors responsible for this complex process. Human mesenchymal progenitor cells were isolated by self selection through monolayer culture and induced to chondrogenic differentiation in a pellet model using culture in a chemically defined serum-free medium. The effects of dynamic compression on freshly isolated and passaged human chondrocytes was also studied.

Results suggest that a defined medium, containing TGFβ, is necessary to induce the re-expression of a differentiated chondrocytic phenotype and the subsequent stimulation of GAG and type II collagen. This thesis highlights a number of areas where knowledge could be improved in relation to the biomechanical events that take place in the repair of cartilage defects and a theory is proposed to explain why marrow techniques lead to a fibrocartilage repair.



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*In memory of Michael Kayser, whose technical help during this project was invaluable and who sadly and unexpectedly passed away at a young age before having time to see the final result.*

*In memory also of my grandparents, David Goldberg and Rachel & Sydney Diamond*

***Healing***
*Time is no healer, like the people say*
*The loneliness grows day by day*
*You laughed with me in good times*
*You cried with me in sad*
*You would have gone without for me*
*You gave me all you had*
*You taught me many things my friend*
*That I may have to do*
*I only wished you taught me*
*How to go on living, without you*

*Rachel Diamond (1917-2002)*

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# CHAPTER ONE – INTRODUCTION & OVERVIEW

## 1.1 Introduction

Articular cartilage covers the articulating surfaces of bones within synovial joints and is crucial for the smooth articulation of joints. It also serves to absorb shock by spreading the load applied to the bony supporting structures below. In most cases articular cartilage is able to carry out its task of strenuous load bearing for a lifetime, however, if it becomes damaged and/or diseased, there is resulting pain and debilitating dysfunction.

Cartilage may become damaged due to congenital defects, direct or indirect trauma, prolonged immobilisation or pathological conditions such as arthritis, infection or inflammation (Buckwalter and Mankin, 1997a). Vath *et al* (1988) reported that cartilage damage was associated with 16% of injuries to the knee that were sufficient to cause a haemarthrosis. Hjelle *et al* (2000) investigated 1,000 consecutive knee arthroscopies and found that chondral or osteochondral lesions (of any type) were found in 61% of the patients and that focal chondral or osteochondral defects were found in 19% of the patients. Similarly Tandogan *et al* (2004) retrospectively analysed 764 patients with anterior cruciate ligament tears who underwent arthroscopy for the first time and found 19% of the knees had one or more chondral lesions. Twyman *et al* (1991) prospectively followed up twenty-two knees in which osteochondritis dissecans had been diagnosed before skeletal maturity and found that 32% had radiographic evidence of moderate or severe osteoarthritis at an average follow up of 34 years.

Due to the lack of vasculature, neural supply and lymphatic drainage together with the low cell density, articular cartilage has limited intrinsic repair potential (Buckwalter and Mankin, 1998). If left untreated, however, the initial damage may

lead to further matrix disruption and the development of progressive degenerative arthritic conditions. Whilst total joint replacements are considered a suitable solution for older patients, they do not provide a satisfactory long-term solution for younger, active patients (Rand and Ilstrup, 1991). In the UK, approximately 5000 patients under 35 present with traumatic damage to articular cartilage each year and in addition, 1,000,000 people under the age of 40 show early arthritic changes, 50% of which will require treatment (Bentley, 1989). Thus the treatment of cartilage damage in the younger patient group provides a major challenge.

The relative failure of many synthetic solutions, has led to the growing interest in the development of cell-based repair systems for solving a number of clinical problems related to articular cartilage. In general two strategies have been considered with regard to restoration of the joint surface. The first strategy is to enhance the intrinsic capacity of the cartilage and subchondral bone to heal using methods such as subchondral drilling (Insall, 1967) or microfracture (Steadman *et al.*, 1998), abrasion arthroplasty (Dandy, 1986), electrical stimulation (Lippiello *et al.*, 1990) and laser treatment (Hardie *et al.*, 1989). These methods usually stimulate the formation of fibrocartilage repair tissue and, as such, have been of only limited success. The second and most promising solution for treatment of the younger patient group lies in the use of tissue engineering approaches where a biological repair is induced.

Two sources of autologous cells have been suggested for tissue engineered repair systems; mature chondrocytes isolated from a cartilage biopsy (Brittberg *et al.*, 1989) or mesenchymal cells derived from bone marrow (Caplan, 1991) or periosteal origin (Rubak *et al.*, 1982a; Rubak *et al.*, 1982b). The use of autologous cells has several drawbacks including the limited number of available cells, the morbidity of

the donor site and damage to the normal tissue around the repair caused by sutures.

The use of multipotential stromal cells of bone marrow origin overcomes some of these problems, but differentiation into mature chondrocytes is essential if repair is to be successful. Some studies have reported differentiation of multipotential stromal cells into chondrocytes within cartilage defects (Wakitani *et al.*, 1994b; Caplan, 1991; Caplan, 1984; Wakitani *et al.*, 1994a). However, the mechanisms involved are unclear and thus it is difficult to modify the techniques to enhance chondrogenesis. The ability to induce chondrogenesis in a defined manner *in vitro* and to design *in vivo* systems which stimulate or maintain chondrogenic phenotype, without the inherent side-effects associated with morphogens, would revolutionise the development of tissue engineered cartilage repair systems.

## 1.2 Cartilage

Cartilage is a soft tissue of mesodermal origin that is present in many structures within the body. Elastic cartilage, which contains many elastic fibres, is found in the nose, pinna of the ear and the epiglottis. Fibrocartilage, which contains more fibrous tissue, is present in the intervertebral discs, the menisci and repair tissue. Hyaline cartilage, however, is the most abundant form of cartilage in the body. It covers the articulating surfaces of bones within synovial joints and is crucial for their smooth articulation, hence it is termed articular cartilage in this context. The name of hyaline cartilage originates from the Greek, hyalos, which means glass, due to its partially translucent appearance (Hunziker *et al.*, 1992). With ageing, however, the tissue becomes progressively more yellow and dull due to dehydration, increased collagen cross-links and accumulation of lipofuscin pigment.

## 1.3 Articular cartilage

Articular cartilage provides joints with excellent friction, lubrication and wear characteristics required for continuous gliding motion, allowing the articulating bones to transmit high loads whilst maintaining contact stresses at low levels. During normal activity articular cartilage is subject to high contact stresses, which may rise to between 10-20 MPa in humans (Hodge *et al.*, 1986). The classic description of mature articular cartilage is that it is aneural, alymphatic and avascular (Caplan, 1984). Due to the poor permeability of the subchondral bone plate, the chondrocytes exclusively derive their nutrients and dispose of their waste products via the synovial fluid.

### Composition of Articular Cartilage

This tissue consists of a sparse population of highly specialised cells, chondrocytes, distributed throughout an abundant extracellular matrix (ECM). Chondrocytes form only 1-10% of the tissue by volume, the remainder being ECM (Stockwell, 1975). The ECM is biphasic. It consists of a liquid phase composed of water, dissolved gases and salts and a solid phase composed of collagen, proteoglycans and non collagenous proteins (Kempson *et al.*, 1973; Mow *et al.*, 1992).

### Spatial Arrangement of Articular Cartilage

The structure and composition of the articular cartilage varies throughout its depth, from the articular surface to the subchondral bone. The differences include alterations in cell shape and volume, collagen fibril diameter and orientation, and proteoglycan concentration. The cartilage is classically divided into four zones (see Figure 1.1) : the superficial zone, the middle or transitional zone, the deep zone and the zone of calcified cartilage (Benninghoff, 1925).

The superficial (tangential) zone forms the gliding surface. The superficial layer is characterised by flattened disc-shaped chondrocytes, low proteoglycan content and densely packed layers of uniform collagen fibres arranged parallel to the surface, which impart the characteristic `hyaline' opacity to cartilage. The superficial layer has been described as a tension resisting diaphragm (Meachim and Stockwell, 1979), manifested by the tendency of articular cartilage to curl when released from the subchondral bone (Broom and Poole, 1982).

The middle (transitional) zone contains large diameter collagen fibres in a less apparent systematic organisation, while the chondrocytes are more rounded in form. The proteoglycan content increases and the collagen fibres decussate to provide an oblique transitional network intermediate between the tangential superficial and radial deep layers.

In the deep (radial) zone the collagen fibres are large and are arranged perpendicular to the joint surface. This zone contains the highest concentration of proteoglycans and the lowest water content. The chondrocytes are spherical and often are arranged in vertical columns of between 4 and 9 cells.

The deepest layer, the zone of calcified cartilage, separates the hyaline cartilage from the subchondral bone and is characterised by small pyknotic cells distributed in a calcified cartilaginous matrix. The chondrocytes undergo hypertrophic changes and are heavily mineralised with hydroxyapatite. Collagen fibres in the deep zone penetrate this zone to anchor the cartilage to the subchondral bone.

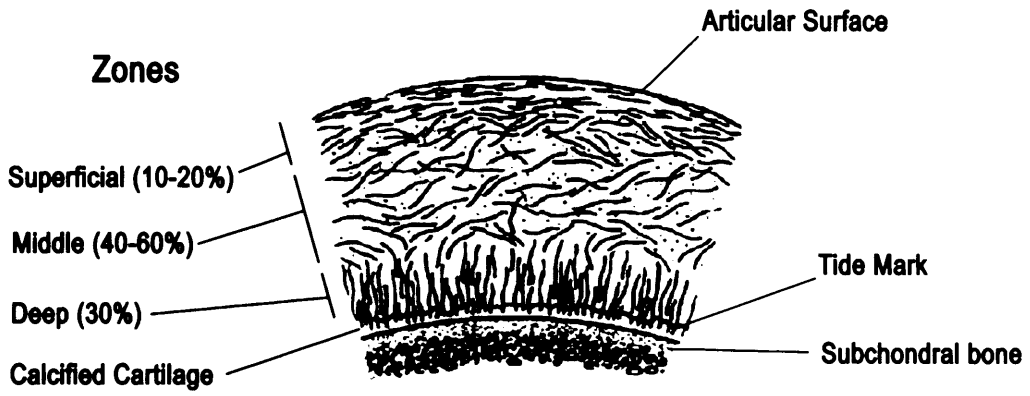



**Figure 1.1.** Schematic diagram demonstrating the zonal structure of articular cartilage, in particular the collagen fibre architecture in a sagittal cross section (adapted from Mow VC, Proctor CS, Kelly, MA: Biomechanics of articular cartilage, in Nordin M, Frankel VH (eds) :Basic Biomechanics of the Musculoskeletal System).

Histological staining with haematoxylin and eosin shows a wavy bluish line (weakly basophilic staining of mineral deposits) called the tidemark, which separates the deep zone from the calcified zone. The tidemark is a site of potential structural weakness along which microfractures can propagate.

**Cellular zones**

In addition to this surface to deep zonal distinctions, the ECM is further subdivided into regions depending on its proximity to the chondrocyte. The pericellular, territorial and interterritorial regions differ in their content and organization of collagen, proteoglycan and other matrix components. The pericellular matrix is a thin layer that completely surrounds the cell membrane. It contains primarily proteoglycans although no collagen fibrils appear to be present. In the middle and deep zones the pericellular matrix is surrounded by a collagen fibre pericellular capsule (Poole *et al.,* 1987). The territorial matrix forms an envelope that surrounds the pericellular matrix and is characterised by thin collagen fibrils that, at the

boundary of the territorial matrix, appear to form a fibrillar network which may protect the cells when the tissue is deformed (Poole *et al.*, 1984).

Benninghoff introduced the concept that the chondrocyte was a 'fluid filled bladder', later known as the 'chondron'' (Benninghoff, 1925). The chondron concept is used to describe the overall functional and metabolic unit of articular cartilage and encompasses the chondrocyte, the pericellular matrix and capsule.

The interterritorial matrix is the largest of the matrix regions and contributes the majority of the material properties of articular cartilage. It encompasses the matrix between the territorial matrices of the individual cells or clusters of cells and contains the large collagen fibres and most of the proteoglycans.

**The Chondrocytes**

The chondrocytes generate the large amount of ECM. In mature articular cartilage chondrocytes occupy less than 10% of the volume yet are still responsible for the maintenance of this matrix. The cells vary in size, shape and metabolic activity but all contain the same organelles for matrix synthesis, including mitochondria, rough endoplasmic reticulum and prominent golgi apparatus. The cytoplasm also contains glycogen, lipids, phospholipids and secretory vesicles. The volume of the cells is greater in the middle and deep zones compared to the superficial zone suggesting a higher metabolic activity. Short cilia extend into the matrix potentially acting as mechanoreceptors (Jensen *et al.*, 2004). Because chondrocytes are isolated by their surrounding matrix, normal cell to cell contact and interactions do not occur. The cells must therefore, sense changes in the matrix either in response to hormonal, biochemical or mechanical signals.

Mitotic activity and cell division is rarely seen in mature articular cartilage. There is, however, evidence that chondrocytes maintain their ability to grow and divide with age (Havdrup and Telhag, 1980) and *in vitro* the cells appear to rapidly multiply in monolayer (Brittberg *et al.*, 1994b).

Chondrocytes are metabolically active and are able to respond to a variety of environmental stimuli, including soluble mediators, such as growth factors, interleukins, drugs, matrix composition; mechanical loading and hydrostatic pressure (Lee and Bader, 1997; Lee and Bader, 1995a; Lee *et al.*, 1997; Yaeger *et al.*, 1997; van Beuningen *et al.,* 1994; Trippel, 1995; Benya *et al.,* 1988).

The chondrocytes plasma membrane expresses MHC antigens I and II. The type-II antigens being strongly expressed in arthritic diseases. H1 and H2 receptors are also present which indicates that mast cells may be able to exhibit a direct effect on the chondrocytes. However, neural impulses and immune responses are not thought to influence the metabolism of the chondrocytes under normal conditions as the tissue is aneural and both monocytes and immunoglobulins tend to be excluded from the tissue by their size (Mankin, 1991; Goldberg and Kresina, 1987; Buckwalter and Mankin, 1997a).

**Other Elements**

**Water (65-80% of wet weight)**

The presence of water permits deformation of the cartilage surface, in response to mechanical loading. The distribution of the water is not homogeneous throughout the cartilage with higher concentrations at the surface (80%) than in the deeper zones (65%). Increased water content leads to increased permeability and decreased mechanical strength. However, in conditions such as osteoarthritis, the

water content increases up to 90%, resulting in significant loss of tissue integrity. Inorganic salts such as sodium, calcium chloride and potassium are dissolved in this water. The water can move through the tissue if a sufficient pressure gradient is applied across the tissue. Frictional resistance coupled with the pressurisation of the water within the ECM are the two basic mechanisms from which articular cartilage derives its ability to support very high joint loads. The flow of water through the tissue and across the articular surface also promotes the transport of nutrients and provides a source of lubrication for the joint.

The affinity of articular cartilage for water derives mainly from the hydrophilic nature of proteoglycans. Indeed the ability of proteoglycans to attract water involves three physicochemical mechanisms:

1. Donnan osmotic pressure, which is caused by the interstitial free floating counter-ions (e.g. $Na^+$, $Ca^{2+}$) required to neutralize the charges on the proteoglycans
2. Electrostatic repulsive forces that are developed between the fixed negative charges along the proteoglycan molecules
3. The entropic tendency of the proteoglycan to gain volume in solution.

In articular cartilage the degree of hydration is determined by a balance of the total swelling pressure, which is the sum of these three effects, exerted by the proteoglycans versus the constraining forces developed within the collagen network surrounding the trapped proteoglycans (Buckwalter and Mankin, 1997a; Mankin, 1991).

**Collagen (10-20% wet weight or 50-60% dry mass)**

Collagens are structural macromolecules which become assembled into fibrils in the ECM. There are many different types and the group of collagens are coded for by

more than thirty genes. Type II collagen represents approximately 90-95% of the total collagen content of articular cartilage, which also contains small amounts of collagen types V,VI,IX,X,XI and perhaps type I (Buckwalter and Mankin, 1997a).

All members of the collagen family contain a characteristic triple helical structure in which three left handed polypeptide helices (α-chains) are twisted around each other to form a right handed super helix (van der Rest and Garrone, 1991). The triple helical conformation is the result of the α-chain polypeptides incorporating a unique repetitive amino acid sequence with the molecular formula (Gly-X-Y)n, with glycine occupying the third position in each amino acid sequence. Proline and 4-hydroxyproline constitute between 10-12% of each of the remaining X and Y residues of the repeating sequence (Thomas *et al.*, 1994). The location of glycine, the smallest amino acid, is an absolute steric requirement for the triple helical structure because a functional group of every third residue occupies the interior of the helix (van der Rest and Garrone, 1991). The presence of hydroxyproline is a requirement for the stability of the collagen helix at body temperature because it allows the formation of intramolecular hydrogen bonds along the length of the molecule. Hydroxylysine participates in the formation of covalent cross links that principally stabilise the collagen fibrillar assemblies.

Cartilage collagens exist both as homotrimers in which all three α-chains are identical (e.g. types II and X) and heterotrimers in which all three α-chains are different (e.g. types VI and IX). Because each α-chain is the product of a distinct gene, cartilage collagens are encoded by at least ten different genes on at least four different chromosomes. In the ECM collagen monomers containing an uninterrupted triple helix align head to tail and side by side in a quarter staggered array, such that overlaps and holes are created in the three dimensional (3-D)

structure, resulting in the characteristic banding pattern seen under electron microscopy (Mankin, 1991).

Some of the collagens, for example type II, appear to be uniformly distributed throughout the cartilaginous matrix, whereas others, for example types VI, IX and XII, may be localised to specific areas. Type II collagen contributes to the framework and gives the cartilage its inherent tensile strength and stiffness. Less is known about the precise roles of collagen types VI and X in articular cartilage, but type VI appears to be localised to the pericellular capsule of the chondrons and may be important in tethering the chondrocyte to its pericellular matrix. Type X collagen is associated with terminal differentiation of chondrocytes (Morrison *et al.*, 1996) and is prominent in the calcified zone of mature joints and may play a role in the mineralization process that occurs just above the subchondral bone. However, during pathological change such as in osteoarthritis, the synthesis of type X collagen becomes more widespread (Stephens *et al.*, 1992). It is of interest to note that surface articular chondrocytes, occupying the uppermost 10-15% of the tissue depth of normal human cartilage, have been shown to initiate *de novo* synthesis of both type X collagen and alkaline phosphatase when maintained in suspension culture (Stephens *et al.*, 1992).

Traditionally it was thought that type I collagen, the most abundant fibrillar collagen in the connective tissues of the body, was not present in mature articular cartilage. However, type I collagen has been demonstrated at the articular surface and in the pericellular environment of hypertrophic chondrocytes in the deeper zones (Wardale and Duance, 1993) of porcine articular cartilage. Such an expression of type I collagen in mature articular cartilage may be species specific, since it has not been found in bovine articular cartilage and although mRNA for type I collagen has been

isolated from mature human articular cartilage, there is no evidence that the mRNA is translated into protein (Kolettas *et al.*, 1995).

**Proteoglycans**

Proteoglycans are complex macromolecules that consist of a protein core to which are linked extended glycosaminoglycan chains. 80-90% of all proteoglycans in cartilage are of the large aggregating type, called aggrecan. They consist of a large extended protein core to which are attached up to 100 chondroitin sulphate and 50 keratan-sulphate glycosaminoglycan chains. The proteoglycans are not homogeneously distributed throughout the depth of the articular cartilage. The superficial zone is rich in collagen and relatively poor in proteoglycans. In the transitional zone, the concentration of proteoglycans increases and they are more homogeneously distributed. In the deep zone the distribution is more variable. Around the chondrocytes in the pericellular matrix there is an approximately two-fold increase in proteoglycan concentration, compared to that in the matrix distant from the cells.

Glycosaminoglycans consist of long-chain unbranched, repeating disaccharide units. Three major types have been found in articular cartilage proteoglycans: chondroitin 4- and 6-sulphate; keratan sulphate; and dermatan sulphate. The chondroitin sulphates are the most prevalent glycosaminoglycans in cartilage, accounting for between 55% and 90% of the total amount, depending on the age of the individual and the presence of osteoarthritis. Hyaluronan is also a glycosaminoglycan, which is not sulphated and hence uncharged. Hyaluronan serves as an anchoring point for the aggrecan molecules and as many as 200 can bind to one chain of hyaluronate to form a large proteoglycan aggregate.

With the exception of hyaluronan, carboxyl (COOH) and/or sulphate ($SO_4$) groups occur along the glycosaminoglycan chains. In solution, these groups become ionized ($COO^-$ and $SO_3$), and in the physiological environment, they require positive counter-ions such as $Ca^{2+}$ and $Na^+$ to maintain overall electroneutrality. This effect is known as the Donnan osmotic effect. The proteoglycans are packed tightly within the tissue and the fixed negative charges repel each other. This repulsion together with the Donnan osmotic effect are the two physical forces which are predominantly responsible for the swelling pressure of cartilage.

Articular cartilage also contains a wide variety of other ECM components including the adhesive substances, chondronectin, fibronectin, laminin, integrin and anchorin which are involved in the interactions between collagen fibrils, proteoglycans and chondrocytes. There are also various cartilage matrix proteins, such as COMP, which are involved in the fine tuning of the matrix properties (Mankin, 1991; Stockwell, 1975; Kempson *et al.*, 1973)

**Metabolism of articular cartilage**

Chondrocytes have some degree of metabolic activity, utilising principally anaerobic pathways to generate ATP required for the synthesis of the matrix components and their distribution within the ECM. The proteoglycan core is synthesised in the rough endoplasmic reticulum (RER) and transported to the golgi apparatus, where the glycosaminoglycan molecules are added prior to secretion into the ECM. The proteoglycans are continually subjected to degradation and are then released from the cartilage, both in the maintenance of normal tissue and in the reparative process. The glycosaminoglycan chains can be identified in blood and urine, the levels of which can indicate catabolic activities. Collagen synthesis follows translation of mRNA to form a polypeptide containing a signal sequence, which

enters the RER. In the RER the signal peptide is glycosylated and hydroxylated to form the triple helix. The procollagen molecule then enters secretory vesicles and is released from the cell. Propeptide sequences are cleaved to release mature collagen molecules which arrange themselves into fibrils. Catabolism of collagens is thought to occur only at a very slow rate in normal cartilage and involves metalloproteinases produced by chondrocytes (Mankin, 1991).

**Development of Articular Cartilage**

It is known that articular cartilage is derived from the lateral plate mesoderm (see Figure 1.2), although relatively little is known about the exact steps that take place during embryogenesis. It has been suggested that the ECM protein tenascin-C is involved in the genesis and function of articular chondrocytes (Pacifici, 1995). It was proposed that tenascin-C may be part of *in vivo* mechanisms whereby articular chondrocytes remain functional throughout postnatal life and yet avoid the endochondral ossification process undertaken by the bulk of chondrocytes located in the metaphysis and diaphysis of skeletal models. More recently a review of the literature suggested that a number of factors might have roles in joint and epiphyseal development (Pacifici *et al.*, 2000). These factors include the homeobox gene Barx-1, the bone morphogenetic protein (BMP) family member GDF-5, the growth factors HGF and PTHrP and the transcription factor ERG.

Using intra-articular tritiated thymidine, two layers of cell proliferation were identified within the articular aspect of the immature rabbit femoral condyle (Mankin, 1962). One layer was the proliferative zone of the ossification centre and the other was located within the growing articular cartilage. The label localised precisely in the region that Clark, studying newborn rabbits, later identified as the forming radial (or

deep) zone (Clark *et al.*, 1997). This author suggested that growth in cartilage thickness occurs primarily through enlargement of the radial (deep) zone.

More recently the growth of articular cartilage was described as being appositional in a mechanism similar to the epiphyseal growth plate (Archer *et al.*, 1994; Archer *et al.*, 1996). Using a panel of specific antibodies, the authors documented the temporal and spatial patterns of some of the proteoglycans in the developing knee cartilage of the marsupial South American opposum (Monodelphis domestica), from parturition to adulthood.

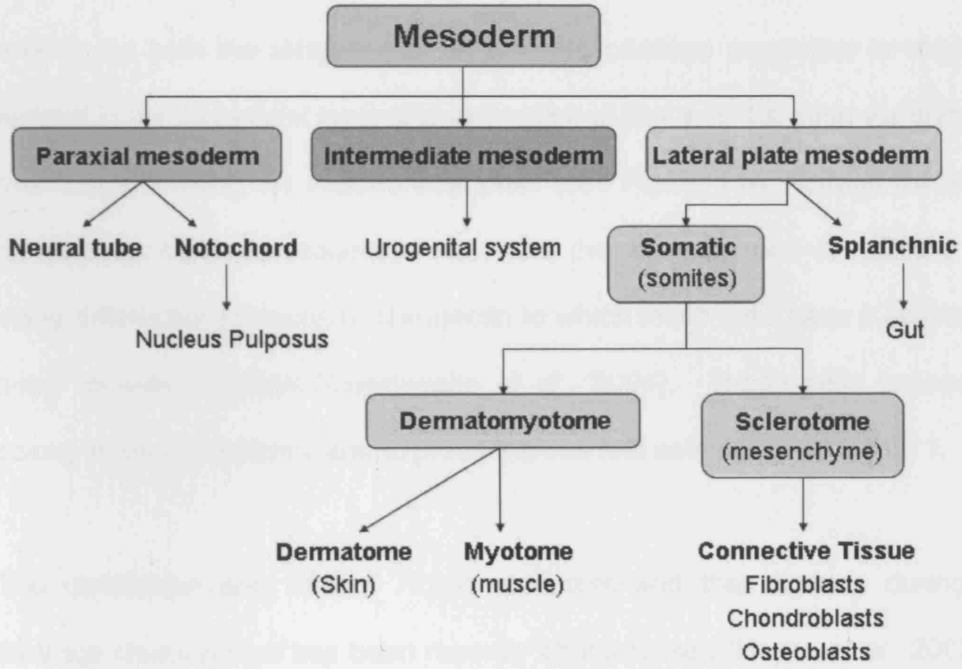



**Figure 1.2.** Schematic diagram illustrating the tissue lineages from embryonic mesoderm.

This work revealed that prior to the formation of the secondary centre for ossification, all factors were distributed evenly throughout the cartilage but after skeletal maturity proteoglycans are preferentially synthesised and elaborated in the upper half of the tissue depth (Archer *et al.,* 1996).

Using the knee joints of the marsupial *Monodelphis domestica*, Hayes *et al* suggested that growth of articular cartilage was appositional rather than through interstitial mechanisms (Hayes *et al.*, 2001). This was carried out through the intra-articular administration of bromodeoxyuridine (BrDU), which blocks chondrocytes proliferation and led to a depletion of the cells in the transitional (middle) zone, and resulted in thinning of the articular cartilage. In order to be appositional there would need to be both the existence of an articular cartilage progenitor or stem cell that resided in the superficial layer and resorption of tissue at the base via endochondral ossification forming the subchondral plate (see Figure 1.3). Indeed the isolation of an articular cartilage progenitor cell from the surface zone of articular cartilage using differential adhesion to fibronectin to which these cells have a high affinity has been recently reported (Dowthwaite *et al.*, 2004). These cells possess a high colony-forming efficiency and express the cell fate selector gene Notch 1.

The distribution and role of Notch receptors and their ligands during articular cartilage development has been recently characterised (Hayes *et al.*, 2003). Notch 1 was expressed by the chondrocytes of the developing articular surface but became increasingly restricted to the deeper layers after birth, whilst expression of this family member was restricted to hypertrophic chondrocytes in the growth plate. The results highlighted the complex Notch signalling interactions that result in the formation of the heterogeneous articular cartilage although the precise mechanisms are yet to be elucidated.

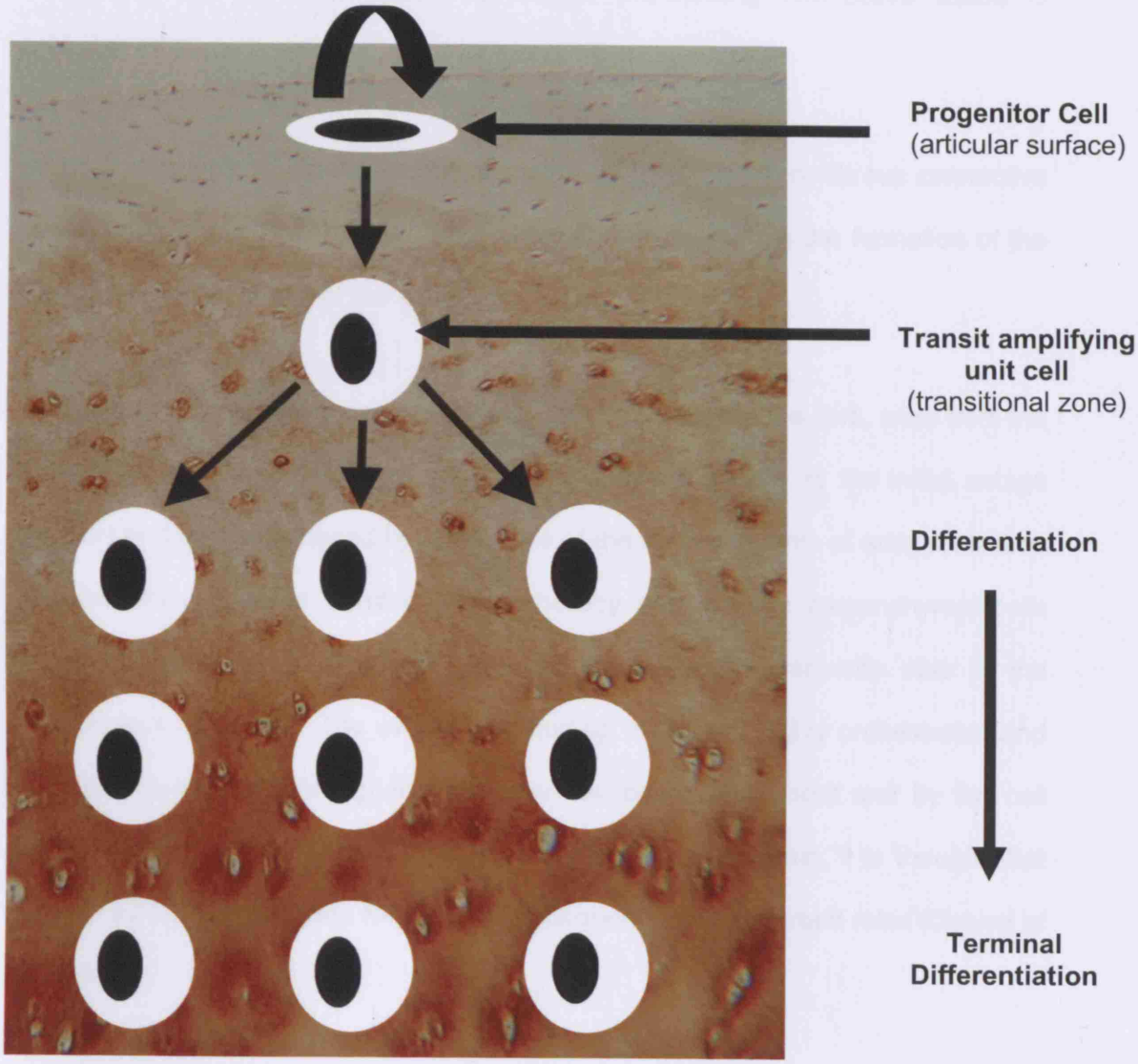



**Figure 1.3.** Illustration of the proposed articular cartilage progenitor cell lineage. Progenitor cells in the articular surface divide to give two daughter cells, one being another progenitor cell and the second being a second transit amplifying unit cell within the transitional zone. The transit amplifying unit cell can undergo further cell divisions along the chondrocyte differentiation pathway. The cells do not migrate through the matrix as it may appear in the diagram rather more, as the articular cartilage grows, through apposition, the relative position of the original transit amplifying cell moves relative to the original progenitor cells which remain at the articular surface (adapted from Hayes *et al* 2001).

There are two kinds of bone formation; intramembranous and endochondral ossification. In both ossification processes, pre-existing connective tissue is replaced by bone.

Intramembranous ossification is the formation of bone on, or in, fibrous connective tissue (from condensed mesenchyme cells) and is involved in the formation of the mandible and flat bones of the skull.

The long bones of the developing skeleton, such as those of the limb, arise from the process of endochondral ossification, where cartilage serves as the initial anlage element and is later replaced by bone. One of the earliest events of embryonic limb development is cellular condensation, whereby pre-cartilage mesenchymal cells aggregate as a result of specific cell-cell interactions, a requisite step in the chondrogenic pathway. The events that lead up to this are highly orchestrated and are likely to be controlled genomically, by the local environment and by the cell itself. Although the precise mechanisms involved are uncertain, it is thought that the cell adhesion molecules N-CAM and N-cadherin have important roles (DeLise *et al.*, 2000).

**Immature Cartilage**

Immature cartilage is more cellular than the mature tissue with little variation in cell density through the zones. Structurally, the surface cells are less flat and are larger. The middle zone is wider and contains a greater number of randomly orientated cells and in the deeper zone the cells are larger with denser shrunken nuclei and large glycogen filled vacuoles. Mitosis is demonstrable by microscopy, whereas in adult tissue mitosis ceases with the development of the well defined calcified zone. As cartilage matures the water content slowly diminishes, the collagen content

increases and the proteoglycan content decreases until it reaches adult steady state proportions.

**Matrix and Chondrocyte Interactions**

The ECM protects the chondrocytes from mechanical damage during normal use of the joint, helping to maintain their shape and phenotype. Throughout life, chondrocytes degrade and synthesize matrix macromolecules although the pathways and mechanisms controlling this process are not fully understood. The various cytokines, including growth factors, play a significant role. In response to a variety of stimuli, such as mechanical loading, chondrocytes synthesize and release cytokines into the matrix, where they may bind to cell surface receptors or may become trapped in the matrix. Activation of the associated receptors may stimulate cellular activity via either autocrine or paracrine mechanisms.

The matrix also acts as a signal transducer for the chondrocytes. There is evidence that a persistent decrease in joint loading or immobilisation of the joint can lead to a decrease in the concentration of proteoglycans and alterations in the mechanical properties of the cartilage. Thus the maintenance of the normal composition of articular cartilage requires a degree of loading and motion of the joint (Buckwalter, 1995). Matrix and cellular deformation are just one of a variety of possible mechanisms of cell mechanotransduction, which also involves streaming potentials, hydrostatic pressure, biochemical, pH and osmotic changes (Knight *et al.*, 1996; Lee and Bader, 1997; Lee and Bader, 1995a; Sah *et al.*, 1989b; Buschmann *et al.*, 1995).

**Effects of growth factors on articular cartilage**

Many growth factors have been shown to have effects on chondrocytes and articular cartilage (Goldberg and Caplan, 1994; Glansbeek *et al.*, 1998; van Osch *et*

*al.*, 1998; Yaeger *et al.*, 1997; Hunziker and Rosenberg, 1994; Sellers *et al.*, 1997; Hsieh *et al.*, 1996). These include transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), fibroblast growth factor, platelet-derived growth factor (PDGF), bone morphogenic proteins (BMP) and growth hormone (GH). Their effects have been reviewed (Trippel, 1995).

Transforming growth factor-β (TGFβ) is a multipotent regulator of cell growth (Frazer *et al.*, 1994) and ECM synthesis. Several distinct isoforms have been described, and three TGFβs 1, 2 and 3, are found in mammalian species (van Beuningen *et al.*, 1994). TGFβ is secreted as an inactive, high molecular weight complex which has to be dissociated prior to functional activation. High levels of active TGFβ have been found in affected joints of osteoarthritic and rheumatoid patients (Fava *et al.*, 1989; van Beuningen *et al.*, 1994), whilst osteoarthritic cartilage appears to be more sensitive to its effects (Lafeber *et al.*, 1993) than normal cartilage. The effects of TGFβ on proteoglycan synthesis remain controversial. Intra-articular injection of TGFβ generally has been shown to stimulate articular chondrocyte proteoglycan synthesis (Glansbeek *et al.*, 1998; van Beuningen *et al.*, 1994; van Beuningen *et al.*, 1993) and induce an increased repair of articular cartilage defects (Abe *et al.*, 1998), however, the induction of synovial hyperplasia and osteophyte formation has been a cause for concern (van Beuningen *et al.*, 1994). *In vitro*, most authors report a stimulation of proteoglycan synthesis by TGFβ (Glansbeek *et al.*, 1998; van Osch *et al.*, 1998; Yaeger *et al.*, 1997; van Beuningen *et al.,* 1994; Benya and Padilla, 1993; van der Kraan *et al.*, 1992b), although there are reports of inhibited proteoglycan metabolism by TGFβ (van der Kraan *et al.*, 1992a; van der Kraan *et al.*, 1992b). Van der Kraan *et al* describes inhibition of proteoglycan synthesis in freshly isolated chondrocytes but stimulation in cells that have undergone monolayer expansion (van der Kraan *et al.,*

1992b). Accordingly, the effect of TGFβ may depend on the stage of differentiation of the chondrocytes. By contrast, Van Osch *et al* has shown that the effects are independent of the stage of differentiation of the cells (van Osch *et al.*, 1998). However, there are significant variations between experimental protocols in studies investigating the effects of TGFβ, and this precludes the elucidation of key underlying mechanisms.

Insulin-like growth factor-I (IGF-I) has been implicated as an important mediator in the induction and maintenance of matrix macromolecules in articular cartilage (Trippel, 1995). In one study a synergistic action was demonstrated between TGFβ and IGF-I on the production of chondrocytic markers by human articular chondrocytes (Yaeger *et al.*, 1997). Indeed this study showed that the effect of TGFβ was profound when combined with insulin or IGF-I but was minimal in their absence.

## 1.4 Biomechanics of Articular cartilage

### 1.4.1 Theoretical Models of Articular Cartilage

During normal joint activities, articular cartilage is subjected to high loads applied statically and cyclically. The maximum joint reaction forces can be as great as 50 times body weight (Seireg and Arvikar, 1973) and so articular cartilage must play an important role in distributing these forces and minimising the stresses on subchondral bone. In order to improve the understanding of how cartilage responds to an external stress and to determine the internal stress/strain state of articular cartilage and chondrocytes, numerous theoretical models of cartilage compression have been developed, evolving through single phasic models to biphasic and later multiphasic models. Detailed descriptions of each of these models have been conveniently summarised (Hasler *et al.,* 1999). The biomechanical properties of

articular cartilage are more easily understood when the tissue is viewed as a biphasic material, composed of a solid and a fluid phase. The solid phase consists of the collagen-proteoglycan matrix and the fluid phase incorporates the interstitial fluid (Mow *et al.*, 1980)

**Solid (fluid independent) phase**

This process is determined by the intermolecular friction between collagen and proteoglycan within the matrix. The specific nature of the interactions between these macro molecules is not fully determined (Maroudas *et al.*, 1968; Maroudas, 1976) but may involve (i) electrostatic interactions resulting from the various charged groups and (ii) the physical entanglements between the collagen fibres and the proteoglycans and between the collagen fibres themselves (Mow *et al.*, 1980).

**Fluid (fluid dependant) phase**

High pressures are needed to move fluid through articular cartilage due to viscous drag effects and the very small diameter pores within the matrix components ($\approx$ 6nm) (Mow *et al.*, 1984). For example, in order to obtain a flow speed of 17.5 $\mu$m/s, a pressure gradient of 1 MPa is required (Simon *et al.*, 1972). The permeability of articular cartilage decreases non-linearly with compression. This strain dependant permeability effect serves to regulate the response of cartilage to compression by preventing rapid and excessive fluid exudation from the tissue with compressive loading and by promoting fluid pressurisation to support the loads. The non linear effect as the tissue is compressed is due to two major factors, namely, the porosity is reduced and the density of negative charges on the proteoglycans increases, thus enhancing the 'water-retaining' environment (Simon *et al.*, 1972).

**Viscoelastic Properties**

Articular cartilage also exhibits viscoelastic characteristics. Accordingly the tissue responds to deforming loads with both viscous and elastic properties and, therefore,

exhibits time-dependant behaviour when subject to a constant deformation or load. When a constant compressive load is applied, the deformation will increase with time, termed "creep", until an equilibrium value is reached (see Figure 1.5a).

During loading, there is an increase in both the interstitial fluid pressure and the matrix compression. However, under constant load, as creep continues, the load support is gradually transferred from the fluid phase (as the fluid pressure dissipates by movement of water) to the solid phase. Typically, for normal cartilage this equilibrium process takes several hours to achieve. At equilibrium, the gradients reduce to zero and load support is provided entirely by the compressed collagen-proteoglycan solid matrix. This is unlikely to occur *in vivo* due to the long equilibrium time and because joints are rarely subject to such prolonged loads. It is likely that in normal articular cartilage, fluid pressurization is the dominant physiological load support mechanism. In fact, it has been estimated that the ratio of load supported by the fluid pressure to that supported by the solid matrix is greater than 20:1 in normal articular cartilage (Simon *et al.*, 1972).

In pathology, such as osteoarthritis, the proteoglycan content of articular cartilage is disrupted. This increases the pore size and thus, water is more mobile. A greater mechanical load is then supported by the solid phase leading to a rapid further degeneration of the joint (Mankin, 1991).

### 1.4.2 Types of mechanical testing of Articular Cartilage

Three experimental configurations are typically used during compression testing on articular cartilage, involving indentation, confined and unconfined compression (see Figure 1.4). Other test configurations that have been used include uniaxial tension and shear testing, although such tests have less clinical relevance.

It is important to emphasise that most of the analytical and numerical methods used to derive material properties of cartilage assume that the tissue is homogeneous and isotropic, which is an oversimplified but necessary step in order to obtain any meaningful data.

**Indentation**

Indentation is one of the most commonly used methods to determine the mechanical properties of articular cartilage. A load is applied perpendicular to the cartilage surface through a plane ended rigid indenter, which may be solid or permeable in nature. The experimental parameters measured in indentation testing involve both creep and stress relaxation. In an ideal creep test, a step load applied to the cartilage produces a transient cartilage deformation and cartilage creeps towards an equilibrium strain with time (Figure 1.5). Similarly, in an ideal stress relaxation test, a constant displacement is imposed on the cartilage. The stress required to maintain the displacement decreases with time until a new equilibrium stress is obtained (see Figures 1.4A and 1.5). The advantage of indentation tests is that they can be used *in vivo* on intact joints and can be used to detect variations in structural properties, such as stiffness across the joint surface.

**Confined Compression**

In confined compression experiments, a cylindrical plug of articular cartilage is fitted inside an impermeable cylindrical confining chamber, so that radial bulging is prevented (see Figure 1.4B). Typically the subchondral bone-cartilage junction is kept intact so that fluid flow across this boundary is negligible. The cartilage surface is loaded using a rigid, porous filter.

**Unconfined Compression**

In unconfined compression, a cartilage sample (usually a circular disc) is loaded between two impermeable platens. Unlike in confined compression, the sides of the

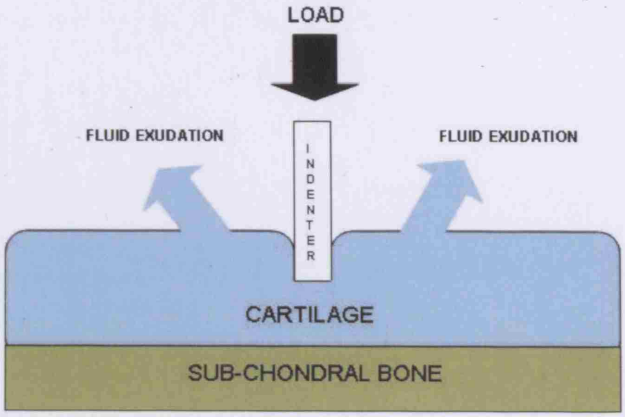
cartilage sample are not restrained and radial expansion is induced by the applied axial compression. Free fluid exudation can occur across the cylindrical boundary surface (Figure 1.4C). These tests allow stress/strain curves to be generated and hence the calculation of Young's Modulus.

### 1.4.3 Permeability

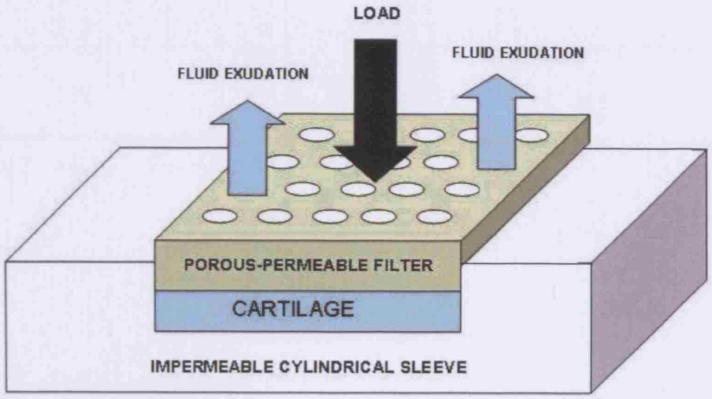
Permeability is a material parameter reflecting the ease in which fluid flows through a porous material and articular cartilage has a very low permeability. Typically, permeability has been measured using two approaches: (1) by curve fitting a biphasic model of articular cartilage to experimental data obtained from compression testing, or (2) by direct measurement in a flow test.

Permeability has been shown to decrease with depth from the articular surface (Maroudas *et al.*, 1968) and with increasing compressive strain and/or increasing pressure. This behaviour is associated with increased drag forces caused by fluid flow through a more compressed matrix with decreased pore size. This decrease in permeability has been interpreted as a mechanical control system to prevent rapid and excessive fluid exudation at high pressures and loads (Mow and Rosenwasser, 1991).

A




B




C

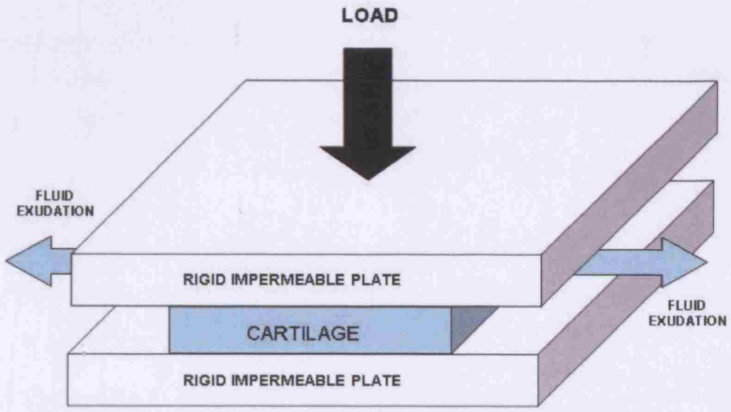



**Figure 1.4.** Types of experimental configurations used for determining the properties of articular cartilage. (A) Indentation (B) Confined Compression and (C) Unconfined Compression.

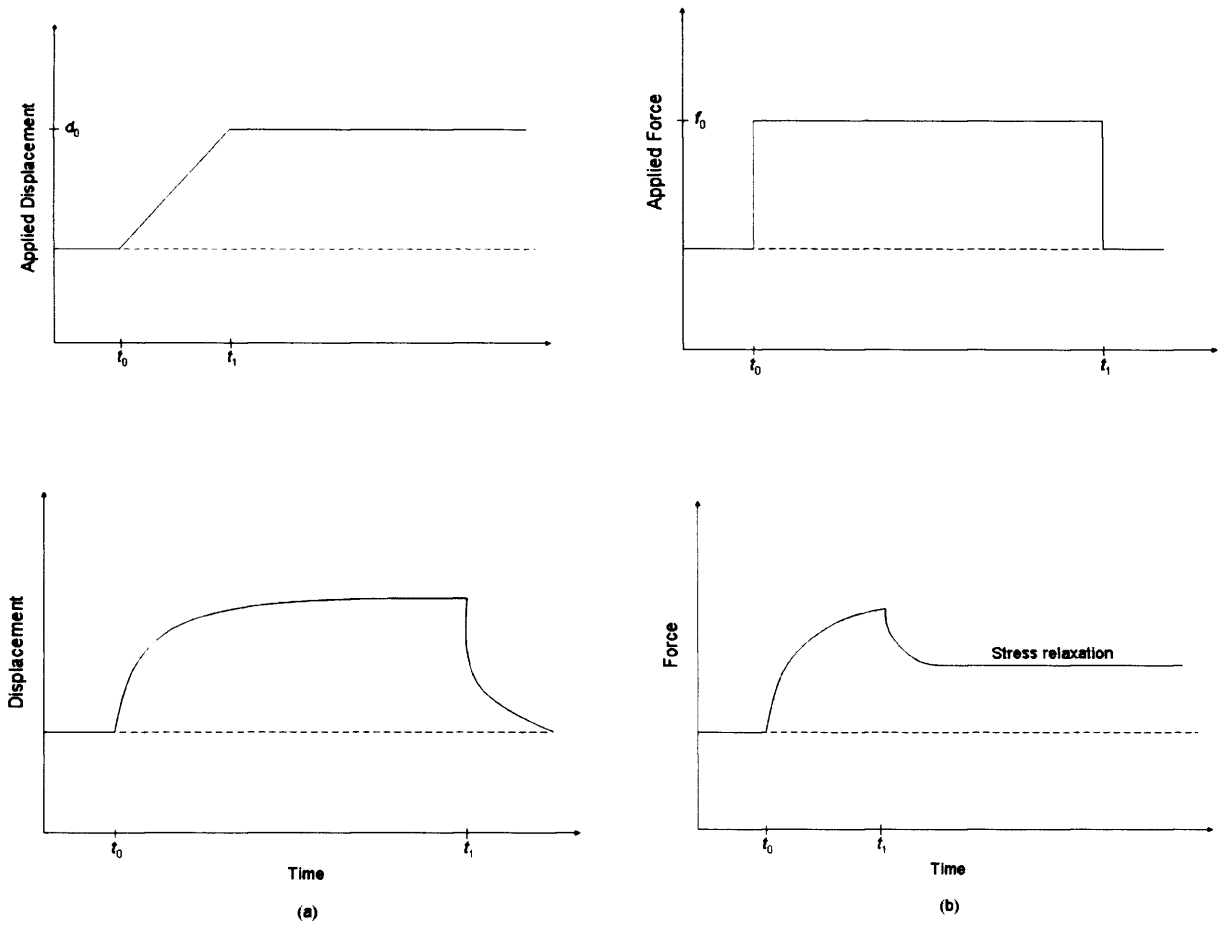



**Figure 1.5.** Schematics of load-deformation behaviour of viscoelastic materials: (a) creep test, (b) stress relaxation (adapted from Mechanical properties of normal and osteoarthritic articular cartilage, Osteoarthritis. Mow & Setton (Mow and Setton, 1998)).

### 1.4.4 Devices for Cartilage Stiffness Measurements in Vivo

There are very few *In vivo* measurements of articular cartilage material properties. They are generally based on cartilage indentation (Dashefsky, 1987) or on electromechanical surface spectroscopy (Sachs and Grodzinsky, 1995), the latter of which involves electrokinetic phenomenon of streaming potentials generated by mechanical compression of cartilage.

*In vivo* measurement of articular cartilage material properties with, typically handheld, indentation devices either employ strain gauge technology or miniature load cells to measure the resisting force of cartilage to an applied deformation. Most of the devices have been tested *in vitro* on excised or enzymatically digested cartilage specimens, although clinical *in vivo* data measured during arthroscopy have been reported (Lyyra *et al.*, 1999; Dashefsky, 1987).

Because the response of articular cartilage to joint loading is dependant on cartilage thickness, a method for assessing the thickness of articular cartilage and stiffness is necessary. Several methods have been used including, stereomicroscopy, needle probe methods, ultrasound and MRI. Although MRI techniques have the potential to be the most accurate, at present ultrasound methods seem to provide the most reliable, non invasive and cost effective method to characterise in vivo cartilage, especially as this can be carried out during indentation testing, by arthroscopic means (Youn *et al.*, 1999).

## 1.5 Damage to articular cartilage

Cartilage damage may occur due to trauma or may be induced by an imbalance between matrix synthesis and degradation. It can be divided into loss of matrix macromolecules and mechanical disruption of the cells and matrix, although the two processes may interact.

### 1.5.1 Loss of Matrix Macromolecules

Loss of matrix macromolecules generally occurs as a result of infection, inflammation, prolonged joint immobilization, haemathrosis and some anti-inflammatory agents. The degradation of proteoglycans can be stimulated, or their synthesis suppressed. If the stimulus is short-lived, then the damage can be reversed and the cartilage restored. However, if it continues, the damage becomes irreversible (Buckwalter and Mankin, 1998). Metabolic causes of loss of matrix macromolecules include rheumatoid arthritis, seronegative spondyloarthropathies, crystalline-induced arthritis and septic arthritis.

**The Process of Osteoarthritis**

Many of the mechanisms responsible for the processes that occur in osteoarthritis remain uncertain. The process of osteoarthritis can be divided into three overlapping stages: disruption of the cartilage matrix, the chondrocytic response to tissue damage and the subsequent progressive loss of tissue (Buckwalter and Mankin, 1998).

In the first stage the macromolecular matrix framework is disrupted and there is a loss of proteoglycans with an associated increase in water content. The reason that the matrix swells is that the proteoglycans, in particular aggrecan, leach out of their confining collagen fibres and attract water. The tissue permeability increases with resultant loss of tensile compressive stiffness and strength. The tissue becomes susceptible to further mechanical damage.

The second stage begins when the chondrocytes detect abnormalities in their environment and release mediators that stimulate a cellular response including the production of cytokines, such as interleukin-1, and intracellular mediators, such as

nitric oxide. Various anabolic and catabolic pathways are activated including metalloproteinase activity, which may induce enzymatic destruction of matrix components (Martin *et al.*, 2004). The balance between the anabolic repair and remodelling processes, and the catabolic damage determines the rate of progression of the disease. Whereas the majority of cases involve a very slow progressive process, in some instances there is rapid deterioration. It is during this second stage that there is some evidence to suggest that operations to alter the mechanical environment of the joint sometimes can stimulate the restoration of the articular surface (Insall *et al.*, 1984; Rinonapoli *et al.*, 1998).

Failure to stabilize or restore the tissue leads to the third stage in the disease where there is progressive loss of articular cartilage with the ensuing clinical symptoms of pain and loss of joint function.

The earliest histological changes seen in osteoarthritis include fraying or fibrillation of the cartilage surface with decreased proteoglycan staining of the superficial and middle zones. In addition, the tidemark may become violated by blood vessels from the subchondral bone. Whether the remodelling of the subchondral bone precedes the degeneration of the articular cartilage or whether the loss of cartilage leads to increased peak stresses on the subchondral bone remains uncertain (Buckwalter and Mankin, 1997b).

**The effect of age and joint-use on the development of osteoarthritis**

With increasing age, chondrocyte apoptosis occurs and the capacity of the remaining chondrocytes to maintain the matrix diminishes with a concomitant loss of proteoglycans. The response of the cells to growth factors decreases and the water content also decreases. There is increased cross linkage between collagens and macroscopically superficial fibrillation in localised areas is visible (Buckwalter

and Mankin, 1998). Although the mechanisms responsible for osteoarthritis remain poorly understood lifelong moderate use of normal joints does not increase the risk. Thus, the degeneration of normal articular cartilage is not simply the result of aging and mechanical wear.

There is a significantly higher incidence of osteoarthritis in older people, with radiographic incidence of 5% in those less than 25 compared to more than 80% of people more than 75 years old (Buckwalter, 1995; Buckwalter and Mankin, 1998). However, not every elderly person suffers from osteoarthritis and since the changes observed in cartilage from older individuals differs from that in osteoarthritis (as described above), the relationship between age and osteoarthritis remains unclear as does the relationship between joint use and joint degeneration. There has been work to show that exercise in normal functioning joints does not lead to or accelerate osteoarthritis. Furthermore, cyclic loading of cartilage stimulates matrix synthesis (Sah *et al.*, 1989b) and can induce an increase in articular cartilage thickness (Stevens *et al.*, 1998). The fact that the patella has one of the thickest layers of articular cartilage supports this notion.

However, high-impact and torsional loads may increase the risk of degeneration of normal joints, and individuals who have an abnormal joint anatomy, joint instability, disturbances of joint or muscle innervation, or inadequate muscle strength or endurance have a greater risk of degenerative joint disease (Buckwalter and Mankin, 1998).

This is confirmed by more recent work which suggested that the cause of the age-related loss of function is progressive senescence of articular cartilage chondrocytes, possibly made worse by oxidative damage caused by excessive mechanical stress (Martin *et al.*, 2004).

In summary, repetitive joint motion in the presence of normal anatomy and joint function is likely to be beneficial for articular cartilage, but excessive stresses and forces are likely to be harmful especially in those individuals who have some genetic or environmental predisposition to osteoarthritis.

### 1.5.2 Osteonecrosis

This is a disabling condition where death of subchondral bone occurs, leaving intact overlying cartilage, which then collapses with underlying bone resorption during revascularisation (Ahlback *et al.*, 1968). It can occur as a sequel to trauma, excessive use of steroids and/or alcohol and in Gaucher's Disease.

### 1.5.3 Osteochondritis Dissecans

If the force of loading is sufficient to cause a fracture of the subchondral bone, osteochondritis dissecans can result. This condition presents as a separation of subchondral bone from the underlying cancellous bone bed. The condition affects two distinct age groups, the young in whom the epiphyses have not yet fused and adults. Its aetiology is poorly understood although trauma is likely to play a major role, perhaps in association with other factors such as developmental or genetic predisposition (Pappas, 1981). In adults the distinction between this condition and an osteochondral fracture is not well defined. Osteochondritis dissecans occurring in the young group has not been shown to lead to osteoarthritis whereas, in adults, the majority of patients develop osteoarthritis in the affected compartment of the knee (Linden, 1977).

### 1.5.4 Mechanical Injury

The principal modes of mechanical injury are fibrillation, chondromalacia and direct trauma (blunt or penetrating). Fibrillation refers to the gradual breakdown of the superficial layer of cartilage due to age and repetitive loading, which causes exposure of the underlying collagen bundles. Chondromalacia is defined as softening of cartilage, which can be caused by decreased amounts of sulphated glycosaminoglycans in the matrix (Outerbridge, 1961).

The main processes involved in the mechanical wear of articular cartilage are interfacial wear and fatigue wear. The former occurs when bearing surfaces come into direct contact, with no intervening fluid lubricant or adsorbed molecules. It can be further subdivided into adhesion and abrasion. Adhesive wear occurs when the bearing surfaces come into contact at focal points. Abrasive wear occurs when a harder material slides across a softer one, scraping it in the process. By contrast, fatigue wear occurs as a result of cyclically repeated deformations of the bearing material. This repeated loading causes repeated stressing of the cartilage matrix, and repeated exudation and absorption of the tissue's interstitial fluid.

**Partial Thickness Injury**

If the injury does not pentrate down to the underlying subchondral bone then no bleeding occurs and it has been suggested that the chondrocytes respond by proliferating and increasing the synthesis of matrix macromolecules at the site of injury (Buckwalter, 1995).

In animal studies, the biochemical results of reparative tissue in response to perpendicular superficial laceration revealed initial presence of dead cells in the traumatised lacunae, immediately after injury. By 24 hours, there was a proliferative

response in the cells adjacent to the necrosed area. This enhanced mitotic activity is associated with increased synthesis of matrix components measured by the incorporation of radio-labelled sulphate and glycine. The increased activity is, however, short-lived and after one to two weeks all values returned to control levels (Mankin, 1991; De Palma *et al.,* 1966). The process falls short however, of any complete repair to the wound.

Experiments in rabbit knees compared tangential superficial lacerations against perpendicular lesions over a two year period using light and electron microscopy (Fuller and Ghadially, 1972). The results did not differ from the perpendicular laceration studies outlined above. Electron microscope findings indicated cell death at the margins of the wound, but also increased mitotic activity in the adjacent surviving cells. Nuclear hypertrophy, increased quantities of rough endoplasmic reticulum and occasional increased numbers of Golgi bodies were noted after injury. However, by six months, the defect remained and except for some minor remodelling at the surface, all healing processes had stopped. The tissue continued to be followed up for two years using electron microscopy. A new layer of homogeneous matrix formed, but by two years, the cartilage surface was found to be almost identical to the appearance immediately after injury.

**Full Thickness Injury**

If cartilage injury extends down to the zone of calcified cartilage, blood vessels in the subchondral bone are disrupted, leading to the formation of a fibrin clot in which red cells and platelets become trapped. There is a release of platelet-derived growth factor (PDGF), transforming growth factor beta (TGFβ), bone morphogenic protein (BMP) and insulin-like growth factor I and II (IGF-I and IGF-II). An inflammatory response ensues. It is hypothesized that within weeks of the injury, mesenchymal stem cells assume the rounded chondrocytic appearance and

synthesize a matrix containing type-II collagens and an abundance of glycosaminoglycans (Osborn *et al.*, 1989).

In studies where full thickness defects have been created it is always found that a fibrous tissue repair ensues, resulting in a tissue that is functionally and mechanically inferior, without the long term durable characteristics of articular cartilage (Hjelle *et al.*, 2000; Steadman *et al.*, 1998; Shapiro *et al.*, 1993; Wakitani *et al.*, 1994b).

**Blunt Injury**

Physiological levels of load do not appear to cause injury to the cartilage, but, when subjected to excessive loads, the cartilage swells, the collagen fibril diameter increases and there is an altered relationship between proteoglycans and collagens (Donahue *et al.*, 1995). These changes may be noted using magnetic resonance images (MRI) after blunt trauma (Ryu *et al.*, 2000). If the injury is of sufficient magnitude to produce fissuring of the cartilage matrix or fractures of the underlying subchondral bone, it effectively becomes a penetrating injury.

Redman et al investigated the different responses of immature articular cartilage to both sharp and blunt trauma in terms of cell death, cell proliferation and matrix synthesis (Redman *et al.*, 2004). They reported a band of cell death adjacent to the lesion edge caused by blunt trauma and recommended the use of sharp precise instruments during the surgical management of cartilage defects.

# CHAPTER TWO – OVERVIEW OF CARTILAGE REPAIR

## 2.1 Classification of articular cartilage defects

The breakdown of articular cartilage has been classified into three stages (Bentley, 1989):

**Grade 1** – Damage restricted to the articular cartilage

**Grade 2** – Exposure of the subchondral bone but no deformation

**Grade 3** – Damage and distortion of the subchondral bone.

In grade 1 defects there is little capacity for repair (Bentley, 1985). In grade 2 damage, exposure of the subchondral bone allows the formation of fibrocartilaginous repair probably from cells in the marrow. Small defects (less than 3mm) often fill completely with fibrocartilage, however larger defects rarely heal (Convery *et al.*, 1972; Pineda *et al.*, 1992; Breinan *et al.*, 1997; Brittberg *et al.*, 1996). Hangody demonstrated that osteochondral defects up to 8mm fill in fully with fibrocartilage, however, this study examined non weight-bearing areas of the joint (Hangody *et al.*, 1998). Accordingly larger defects may fill in with mechanically-inferior fibrocartilage tissue that subsequently degrades in response to load. In both grade 1 and 2 changes, a cartilage repair procedure may help prevent progression to osteoarthritis. In grade 3 damage the only available options are osteochondral transplants or total prosthetic replacement.

### Clinical Classification of Cartilage Defects

Classification systems grading articular cartilage damage were initiated by the work of Outerbridge when describing chondromalacia of the patella (Outerbridge, 1961). The advent of arthroscopy, subsequently gave rise to a less invasive means of diagnosis and many other surgeons have devised their own classifications, often loosely based on the Outerbridge's earlier classification (Bentley and Dowd, 1984;

Terry *et al.*, 1988; Insall *et al.*, 1976; Goodfellow *et al.*, 1976; 1977; Ogilivie-Harris and Jackson, 1984; Johnson-Nurse and Dandy, 1985; Levy *et al.*, 1996; Noyes and Stabler, 1999). None however, have been universally accepted and the classification of Outerbridge remains the most commonly used. In 1988, however, a description based on arthroscopic assessment appeared to be an improvement on that of Outerbridge in that it is easy to use and allows one to follow the natural progression of the lesions (Bauer and Jackson, 1988).

The composition of the tissue is as important as its appearance and so probing the tissue is an essential part of the assessment of cartilage defects. Some centres now have the facility to perform pressure indentation studies during an arthroscopy, which provides some quantitative data on the mechanical properties of cartilage defects and their repair.

The various classification systems necessarily include subjective errors and it has been impossible to accurately compare the results between studies. Even if only one classification system was used many patients have repeated procedures to a cartilage defect that will alter its classification.

**Outline of the major clinical classifications of cartilage defects:**

1) **Outerbridge Classification** (Outerbridge, 1961)

Outerbridge classified the macroscopic appearances into 4 grades:

**Grade 1** – softening and swelling of cartilage

**Grade 2** – fragmentation and fissuring <0.5 inches (13mm)

**Grade 3** – same as grade 2 but > 0.5 in inches (13mm)

**Grade 4** – erosion of cartilage down to bone

Bentley subsequently modified this grading (Bentley and Dowd, 1984; Bentley, 1970) allowing a distinction between depth and size. Grade I is less than 5mm, Grade II is 5-10mm, Grade III is 10-20mm and Grade IV is 20-30mm. Each grade is provided with a suffix, a or b, depending on whether they are partial or full thickness lesions, respectively.

**2) Bauer and Jackson Classification** (Bauer and Jackson, 1988)

Based on a study of 167 chondral lesions in 140 knees Bauer and Jackson identified six different types of lesions of the articular cartilage. Type I, linear crack; type II, stellate fracture; type III, flap; type IV, crater; type V, fibrillated; and type VI, degrading (see Figure 2.1).

**3) International Cartilage Repair Society (ICRS) Classification**

The ICRS Clinical Cartilage Injury Evaluation system was developed during ICRS Standards Workshop in January 2000. This evaluation system consists of two parts; a patient part and a surgeon's part. The patient's part is the ICRS Injury questionnaire and the IKDC Subjective Knee Evaluation Form-2000. The surgeon's part includes the ICRS Knee Surgery History Registration, the IKDC Knee Examination form-2000, the ICRS- Articular cartilage injury mapping system, the ICRS-Articular cartilage injury classification, the ICRS-Osteochondritis dissecans classification and the ICRS-Cartilage Repair Assessment system. Although providing a thorough assessment, the system is complex and thus likely to be practical only in trial settings.

**Methods for assessment of Articular Cartilage Defects**

The success of a cartilage repair procedure can be measured using four methods:

1) **Clinically** – This can be a subjective or objective assessment. Either using an existing grading system such as the International Knee Documentation Committee Subjective Knee Form (Irrgang *et al.*, 2001), The Knee injury and Osteoarthritis Outcome Score (KOOS) (Roos and Lohmander, 2003), the visual analogue score (VAS), the modified Hospital for Special Surgery (HSS) score (Table 2.1) or the author's own. Brittberg for example, graded his patients as excellent (no pain, swelling or locking with strenuous activity), good (mild aching with strenuous activity, but no locking or swelling), fair (moderate pain with strenuous activity and occasional swelling, but no locking) and poor (pain at rest, swelling and locking) (Brittberg *et al.*, 1994b).

2) **Arthroscopically** - This allows the assessment of the general appearance of the repair, the incorporation with adjacent cartilage and the feel or consistency of the repair (indentation tests).

3) **Histologically** – A biopsy is taken which allows a histological assessment. identifying the normal architecture of articular cartilage including the attachment to subchondral bone.

4) **Radiologically** – Plain X-rays and Magnetic Resonance Imaging (MRI).

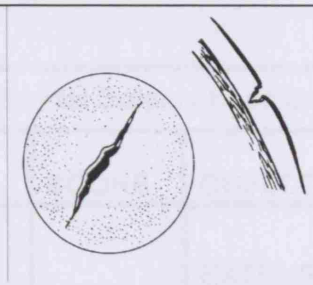
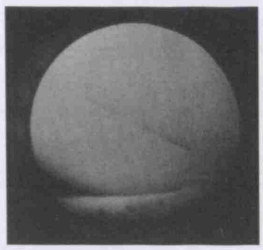
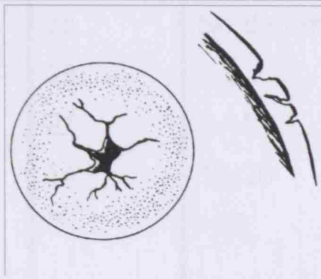
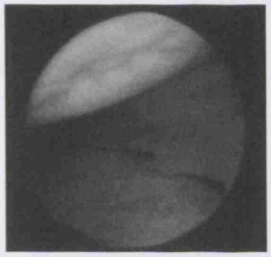
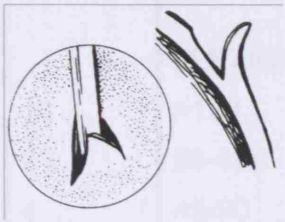
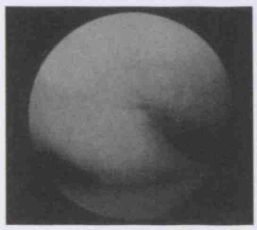
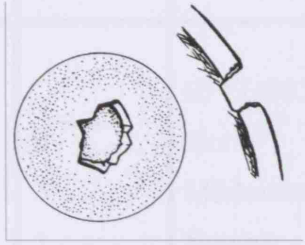
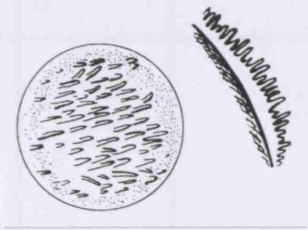
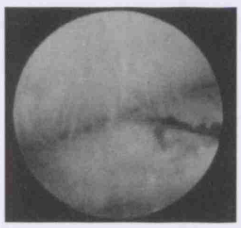
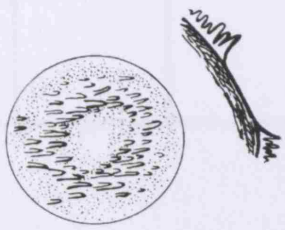
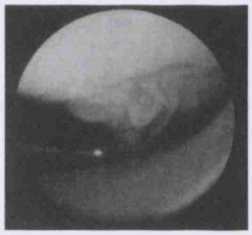
| | | |
|---|---|---|
| **TYPE I – Linear crack**<br>• 3%<br>• Usually split thickness<br>• Result of shearing force<br>• Rotation |  |  |
| **TYPE II – Stellate**<br>• 28%<br>• Mainly weight bearing<br>• Direct trauma<br>• Rotation |  |  |
| **TYPE III – Flap type**<br>• 17%<br>• Mainly weight bearing<br>• Rotational |  |  |
| **TYPE IV – Crater**<br>• 11%<br>• Mainly weight bearing<br>• Direct trauma<br>• In children would be osteochondral fracture |  |  |
| **TYPE V – Fibrillation**<br>• 18%<br>• Partial Thickness<br>• Early degeneration |  |  |
| **TYPE VI – Degrading**<br>• 23%<br>• Older age group<br>• Localised OA |  |  |

**Figure 2.1** Bauer and Jackson classification of articular cartilage defects (adapted from "Chondral Lesions of the Femoral Condyles : A System of Arthroscopic Classification", 1988) (Bauer and Jackson, 1988).

**Table 2.1** - Modified Hospital for Special Surgery Knee Score (Ghazavi *et al.*, 1997)

| SUBJECTIVE FACTORS | SCORE | OBJECTIVE FACTORS | SCORE |
|---|---|---|---|
| PAIN INTENSITY | | EXTENSION LACK (degrees) | |
| None | 35 | No deformity | 10 |
| Mild | 28 | < 5 | 7 |
| Moderate (occasional analgesics) | 21 | 5-10 | 4 |
| Severe | 14 | 10-20 | 2 |
| At Rest | 0 | >20 | 0 |
| INSTABILITY | | FLEXION (degrees) | |
| None | 10 | >120 | 20 |
| Occasionally | 7 | 90-120 | 15 |
| Moderate (with ↓ activity) | 4 | 45-90 | 8 |
| Severe (using brace) | 0 | <45 | 0 |
| WALKING AIDS | | EFFUSION | |
| None | 5 | None | 10 |
| Cane | 3 | Moderate | 5 |
| Crutches | 1 | Severe | 0 |
| Walker | 0 | | |
| WALKING DISTANCE | | | |
| > one mile | 10 | | |
| 0.5-1.0 mile | 6 | | |
| < 0.5 mile | 3 | | |
| Inside house | 1 | | |
| Confined to bed | 0 | | |

## 2.2 Treatment of Articular Cartilage Defects

The aims of treatment are to reduce symptoms and prevent or delay the progression of osteoarthritis. The treatment strategies can be divided into four categories:

1) **Restore** the defect by enhancing the intrinsic capacity of the cartilage and subchondral bone to heal, for example, with the use of growth factors, laser therapy and/or drugs.
2) **Regenerate** the surface by transplanting cells capable of chondrogenesis. This may involve chondrocytes, periosteal grafts or *in-vitro* differentiated mesenchymal progenitor cells.
3) **Relief** of the forces acting on the damaged cartilage. This can be achieved with orthotics, weight loss, walking aids or an osteotomy.
4) **Replace** the damaged cartilage with an autograft, an allograft or an artificial material.

The main thrust of this thesis is to study regeneration of the surface using cell based techniques. A detailed analysis of the other categories is beyond the scope of this thesis, however, the author believes that a brief discussion on the remaining categories is essential as they raise a number of important issues:

**Pharmacological Agents**

The effects of a number of drugs with respect to cartilage healing have been studied. The drugs have been administered through a number of routes, including systemic, intra-articular or local administration.

There had been some suggestion that corticosteroids could enhance cartilage healing (Olah and Kostenszky, 1976), however, many more reports have shown

them to impair the normal physiology of articular cartilage and induce arthropathy (Salter *et al.*, 1967; Mankin and Conger, 1966; Salter *et al.*, 1967; Bentley, 1969).

Hyaluronan is a viscoelastic supplement, which is administered by multiple intra-articular injections in an attempt to improve the joint lubrication and elasticity of the synovial fluid and thereby reduce pain. Other potential benefits include stimulation of endogenous hyaluronan synthesis by synovial cells and proteoglycan synthesis by chondrocytes, inhibition of the release of chondrodegradative enzymes, an effect on the nocioceptive receptors in joint tissues and a scavenging function on oxygen free radicals (Iwata, 1993; Tomford, 1998). Several placebo-controlled trials have been conducted, which indicate a beneficial effect with effects lasting for up to 26 weeks (Dougadous *et al.*, 1993; Altman and Moskovitz, 1998; Lohmander *et al.*, 1996; Huskisson and Donnelly, 1999). Equally there are many trials that have failed to demonstrate significant beneficial effects (Henderson *et al.*, 1994; Dahlberg *et al.*, 1994). In the absence of any alternatives, viscosupplementation with high-molecular-weight hyaluronan can be considered for use in patients with knee OA, who have ongoing pain or are unable to tolerate conservative treatment or joint replacement.

Chondroitin Sulphate & Glucosamine are oral supplements taken either singly or in combination and have been alleged to act as essential substrates necessary for the production of proteoglycans. In veterinary medicine, these compounds have been used for years as a treatment for arthritic symptoms. Human trials have been conducted which show beneficial effects lasting for several months when compared to placebo (Bourgeois *et al.*, 1998; Bucsi and Poor, 1998). There is insufficient evidence available to show any halt in the natural history of the disease. Reginster *et al* assessed the effects of glucosamine sulphate on symptoms and long-term progression of osteoarthritis. They performed a randomised, double-blind placebo

controlled trial, in which 212 patients with knee osteoarthritis were randomly assigned 1500 mg sulphate oral glucosamine or placebo once daily for 3 years (Reginster *et al.*, 2001). There was no significant joint-space loss in the 106 patients on glucosamine sulphate, whereas the 106 patients on placebo had a progressive joint-space narrowing. Similarly, symptoms in the treatment group were significantly better than the controls. The conclusion of the study was that glucosamine sulphate could be a disease-modifying agent in osteoarthritis.

**Replacement of Articular Cartilage**

Replacement of articular cartilage can range from the local substitution of a scaffold (eg carbon fibre mesh) into the defect, to allografting a part of the joint, through to total joint replacement.

**Carbon Fibre – Matrix Support Prosthesis**

Carbon fibres were originally developed for the aerospace industry because of their strength and stiffness relative to density. After implantation in living tissue the carbon fibres become rapidly invaded by fibrous tissue. A system of carbon fibre pads and rods were developed for use in cartilage defects and reported success rates of 70-80% for pain relief (Minns *et al.,* 1982; Muckle and Minns, 1979; Minns and Muckle, 1989).

Brittberg *et al* reported a prospective follow up of 37 patients with early knee osteoarthritis (Outerbridge Grade IV). Carbon fibre pads were used for defects on the patella and rods were used for femoral condylar defects. There were 15 isolated femoral condylar defects, 3 patellar defects and 19 knees had both patella and femoral condylar defects. Early movement was encouraged postoperatively and at an average follow up of four years, 83% of the patients were rated as good

or excellent. Arthroscopy was performed on 20 patients at one year, which showed the defects to be filled with fibrous tissue with no signs of synovitis (Brittberg *et al.*, 1994a). Similarly Pongor *et al* reported an independent review of 96 patients treated by either carbon fibre pads or rods. At follow up of between 9 months to 5 years, both objectively and subjectively about 70% were improved by the procedure (Pongor *et al.*, 1992).

In contrast, Meister *et al* (1988) looked at the role of carbon fibre implants for patella defects in 27 patients with a mean period of follow-up of 33 months. Overall there were 13 poor results (48%) and nine patients subsequently had a patellectomy. Of the remaining patients only 26% had a good or excellent result. They noted consistent seeding of the joint with carbon-fibre debris and a giant-cell histiocytic reaction in the synovium. The use of carbon fibre implants has now been largely abandoned.

### 2.2.1 Osteochondral transplantation

#### Allografts

The use of osteochondral allografts for the repair of large defects of articular cartilage has been explored since the turn of the 20$^{th}$ Century (Tuffier, 1901; Lexer, 1908b), however, problems with rejection have hindered their clinical use (Mankin *et al.*, 1983). The first joint transplants were reported in 1908 (Lexer, 1908b; Lexer, 1908a). By 1925, Lexer had performed 25 total joint allografts with about fifty percent permanent cures mainly in the knee joint but also in the finger joints (Lexer, 1925). In recent years advances with our immunological understanding have led to a resurgence of interest in using allografts. The survival of the allograft after transplantation is key to the success of the procedure. Articular cartilage is thought to be "immunologically privileged" (Langer *et al.*, 1977; Langer and Gross, 1974)

and so although antibodies are produced, the rejection is weak, presumably because they cannot reach the chondrocyte due to the surrounding matrix (Elves, 1976). The transplantation of bone, however, acting as the scaffold, should be associated with an immunological response, although the previously mentioned studies have failed to show this to be a significant problem in clinical practice.

Fresh tissue refers to tissue kept no longer than 72 hours after death, which obviously imposes a time constraint on the use of the graft. To avoid this problem an alternative is to either freeze the allograft or preserve it in culture medium (Black *et al.,* 1979). The chondrocyte viability and biomechanical properties of allografts have been reported to be maintained after storage in culture medium at 4°C for up to 28 days (Black *et al.*, 1979; Kwan *et al.*, 1989; Sammarco *et al.*, 1997). More recently the storage of tibial allografts at either 37°C or 4°C were compared for up to 84 days in medium (White *et al.*, 1999). At 37°C there was no significant depletion of glycosaminoglycan and the allografts maintained 66% of their initial mechanical stiffness, whereas at 4°C results were much poorer.

Freezing would allow even longer storage and may reduce antigenicity (Stevenson *et al.*, 1989), but may cause cell death (Rodrigo *et al.*, 1987; Malinin *et al.,* 1985). Cryopreservative agents, such as glycerol and dimethyl sulphoxide (DMSO), may be of limited use because they do not penetrate deep enough into the tissues (Schachar *et al.*, 1977). Despite this, Flynn reported on 15 patients (17 knees) who received fresh-frozen allografts treated with DMSO and stored at -80°C. In this group of patients with osteonecrosis of one of the femoral condyles, a successful result was achieved in 70% at an average follow up of 4.2 years (Flynn *et al.*, 1994). A further study also reported a series of 15 osteochondral allografts, eight of which were cryopreserved at -80°C (Marco *et al.*, 1993). They found no difference

clinically or radiologically between the fresh and frozen allografts after 3.2 years of follow up.

Ghazavi *et al* reported on 123 patients (126 knees) with post-traumatic osteochondral defects treated by fresh small-fragment osteochondral allografts. At a mean follow-up of 7.5 years (2 to 22), success was achieved in 85%. Failure was attributed to factors such as malalignment, age over 50 years, bipolar grafts (both femur and tibial surfaces are replaced) and workers' compensation cases. Survivorship analysis showed satisfactory results of 95% at 5 years, 77% at ten years and 66% at 20 years (Ghazavi *et al.*, 1997).

Other authors present similar success rates (Locht *et al.*, 1984; Meyers *et al.*, 1989; Convery *et al.*, 1991; Garrett, 1994). One interesting observation is that in patients with unicompartmental degenerative arthritis involving both the tibia and femur, results were uniformly poor (Meyers *et al.,* 1989).

Salai reported an average of 17.1 years follow up on 6 patients who underwent fresh osteochondral allografting to the tibio-femoral joint. The authors changed their policy from using no fixation to holding the fragment with K-wires following graft displaced in one patient. Two patients required proximal tibial osteotomy at 16 and 23 months for mal-alignment. All patients continued to progress at the last reported follow-up (Salai *et al.*, 1997). An interesting observation from the authors' was that the long-term results were more promising than the short-term results.

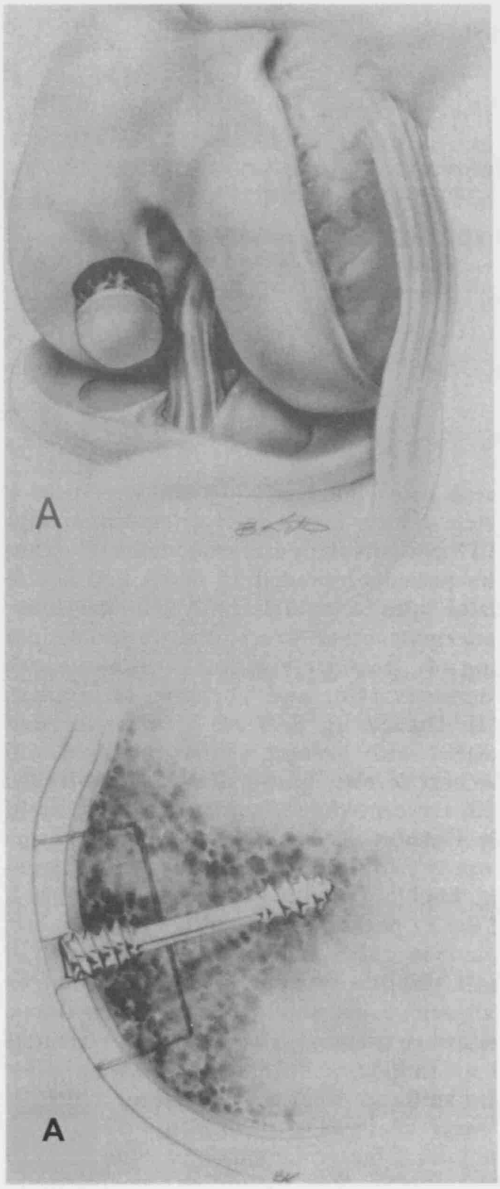
The method of fixation of the graft varied among studies. Some groups used a press-fit technique and only if this was found to be unstable would they supplement it with screw or pin fixation (McDermott *et al.*, 1985; Meyers *et al.,* 1989). Other

studies always used fixation (see Figure 2.2) (Ghazavi *et al.*, 1997; Locht *et al.*, 1984; Garrett, 1994; Meyers *et al.*, 1989; Flynn *et al.*, 1994).

Additionally, the postoperative management varied between studies, some using continuous passive movement immediately following surgery (Ghazavi *et al.*, 1997; Salai *et al.*, 1997; Garrett, 1994; Meyers *et al.*, 1989; Flynn *et al.*, 1994) and other immobilising the leg in a plaster (Locht *et al.*, 1984; Flynn *et al.*, 1994). Weight bearing in most groups was avoided until there was evidence of bony union (Salai *et al.,* 1997; Ghazavi *et al.*, 1997; Garrett, 1994; Meyers *et al.*, 1989; Flynn *et al.,* 1994), which usually occurred at 6 weeks with small grafts and about 4 months with grafts to the tibial plateau. Other groups allowed early partial weight bearing (McDermott *et al.*, 1985).

To assess the viability of the chondrocytes in the allografted cartilage, a study used biopsies at 12, 24 and 41 months (Czitrom *et al.*, 1990). Using $^{35}SO_4$ and $^{3}H$-Cytidine autoradiography, the authors reported a viability of between 69%-99%. Even in failed grafts 66% of the grafts had viable chondrocytes. Other studies have also found chondrocytes to be viable even in failed allografts after periods as long as twelve years (Ghazavi *et al.*, 1997; Kandel *et al.*, 1985).

Although infection, including viral transmission is a potential risk, screening is now performed routinely. There have been two reported cases of HIV transmissions both from frozen femoral head allografts donated in 1984 and 1985 respectively (Tomford, 1985). Since the introduction of screening in the mid-1980s no further cases have been reported.



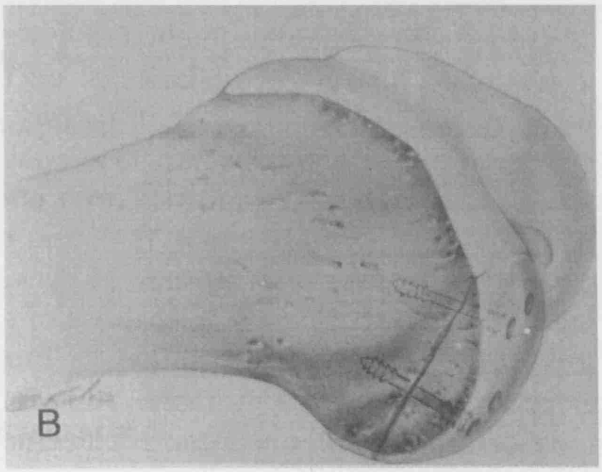




**Figure 2.2** Illustration of some of the different types of allograft (A) an allograft placed into an osteochondral defect of the medial femoral condyle and held in situ by a screw without a head that embeds within the articular cartilage. (B) an allograft to replace the entire lateral femoral condyle, held in situ by four screws (Garrett, 1994).

**Autografts**

In 1952 Wilson and Jacobs described an operation where a patellectomy was performed and the lateral facet of the resected patella was used as an autograft to reconstruct a depressed fracture of the lateral tibial condyle. A review of the original work confirmed the success of the technique and also provided histological evidence of incorporation of the transplanted bone as indicated in Figure 2.3 (Jacobs, 1965). Other authors using a similar technique have reported similar success (Karpinski and Botting, 1983; Campanacci *et al.*, 1985; Outerbridge *et al.*, 1995).

The first description of the transplantation of a small ostechondral plug from one area of the joint to another came from a Japanese group who successfully performed the procedure on two patients (Yamashita *et al.*, 1985).

Hangody, a Hungarian surgeon, using this data together with his own animal studies, developed a procedure known as Mosaicplasty (Hangody *et al.*, 1997; Hangody *et al.*, 1998). Since the early 1990's, the technique has been performed on hundreds of patients in Hungary and other centres in the USA, Canada and Europe to treat defects of the knee or the dome of the talus. In this technique, which can be performed either arthroscopically or through a minimal incision, small osteochondral plugs are taken from the non weight-bearing periphery of the patellofemoral joint. They are implanted in a mosaic fashion into defects in the weight bearing parts of the knee. Using a combination of 2.7 mm, 3.5 mm and 4.5 mm grafts the recipient site will be filled with 60-80% transplanted hyaline cartilage. Fibrocartilage "grouting" growing upward from the prepared cancellous bed completes the mosaicplasty. Approximately 25% of patients have defects considered suitable for arthroscopic mosaicplasty. The patients are kept non weight-bearing for 2 weeks, partial weight-bearing for a further 4 to 6 weeks. At 3 months normal daily activities are allowed and by 9 months full return to sporting activities, including football, are allowed under close supervision. Theoretical and

practical considerations suggest that the ideal diameter of the defect is between 1 and 4 $cm^2$. Donor-site availability and other technical circumstances in the main determine these limitations. Usually, both of the patellofemoral peripheries allow graft harvest for defects of 3 to 4 $cm^2$ in size. Under certain conditions, the mosaicplasty can be used as a salvage procedure for defects as large as 8 to 9 $cm^2$, but such extension of the indication can result in a higher rate of donor-site morbidity.

Using a modified HSS score, Hangody graded 91% of 57 patients as good or excellent at a follow up of more than three years (Hangody *et al.*, 1998). In this group 8 patella defects were included. During second-look arthroscopy in 19 patients with twelve biopsies, the transplanted cartilage remained hyaline in character and the bonding between recipient and donor plugs was filled in by fibrocartilage. Two of the 19 patients, however, had evidence of surface fibrillation.

Kish *et al* reported on 52 competitive athletes undergoing mosaicplasty, with an average follow up of 26.5 months (Kish *et al.,* 1999). It is presumed that some of these patients had been included in the previous data set, however, no defects of the patella were reported in this group. The authors divided the results into two groups. In the younger group, under 30 years old, 90% returned to full competition, 7% at a reduced level and 3% changed to a less vigorous sport. In the group aged over 30, twenty three percent returned to competition, 70% at a reduced level, 2% changed their sport and 4% retired. No morbidity appeared to arise from the harvest sites, which filled in with fibrocartilage over several months. Concomitant procedures, such as ACL reconstruction or meniscal repair, did not seem to affect the outcome. In this paper they highlight some case examples of problems they encountered such as plug subsidence. The authors also emphasise the need for patient selection and education as early weight bearing against advice will lead to a poor result.

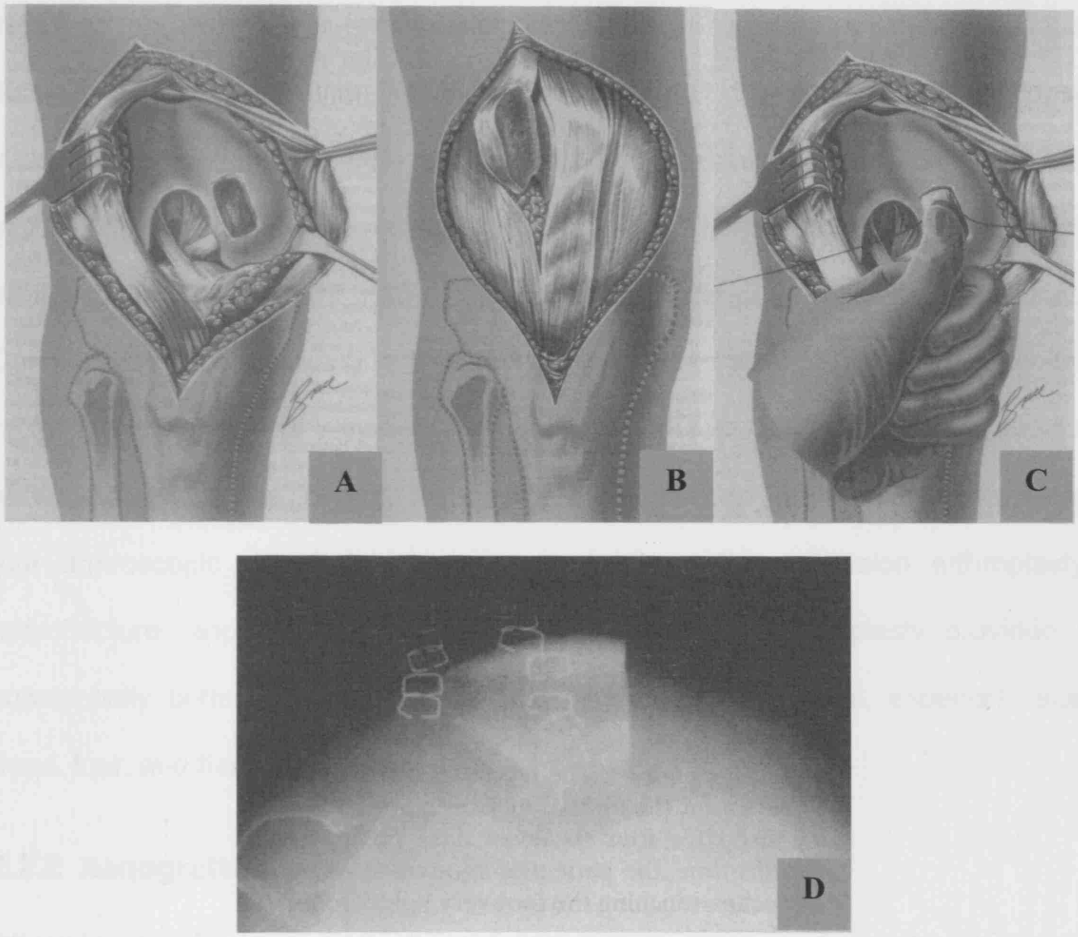



**Figure 2.3** A procedure carried out by Outerbridge where an autograft harvested from the lateral half of the patella (B) was used to graft a defect (A) of the medial femoral condyle (C) (Outerbridge, 1995). In (D) a postoperative radiograph demonstrating the result (Jacobs, 1965) is shown.

More recently, the ten year clinical experience on 831 patients undergoing autologous osteochondral mosaicplasty was documented (Hangody and Fules, 2003). Using clinical assessment, imaging techniques, arthroscopy, tissue indentation measurements and biopsy the authors showed good-to-excellent results in 92% of the patients with femoral condylar defects, 87% of those treated with tibial defects, 79% of those with patellar and/or trochlear defects, and 94% of those treated with ankle talar defects. Long-term donor-site disturbances, assessed with use of the Bandi score, revealed just 3% morbidity. Of those who underwent arthroscopic assessment 83% (n=83) had congruent gliding surfaces. Complications of the surgery included four deep infections (0.5%). The average size of the defects was not reported (Hangody and Fules, 2003). In this paper, the authors refer to a multi-centre, prospective study involving 413 patients comparing four arthroscopic resurfacing techniques: Pridie drilling, abrasion arthroplasty, microfracture, and mosaicplasty. They claimed that mosaicplasty provided a substantially better clinical outcome than the other techniques, especially after three, four, and five years (Hangody and Fules, 2003).

### 2.2.2 Xenografts

Attempts at using xenografts have failed due to what appeared to be intense immunololgical reaction. The literature on these procedures was summarised by Bentley (Bentley, 1972) and despite a report of the successful implantation of bovine menisci into rabbits (Bentley, 1989), the use of xenografts does not appear to be an acceptable option for clinical use.

### 2.2.3 Artificial Transplants

The difficulty with artificial substitutes is achieving a good attachment to bone. A Japanese group developed an artificial material called polyvinyl alcohol (PVA) hydrogel, which may be used as a replacement for articular cartilage (Oka *et al.*, 1997).

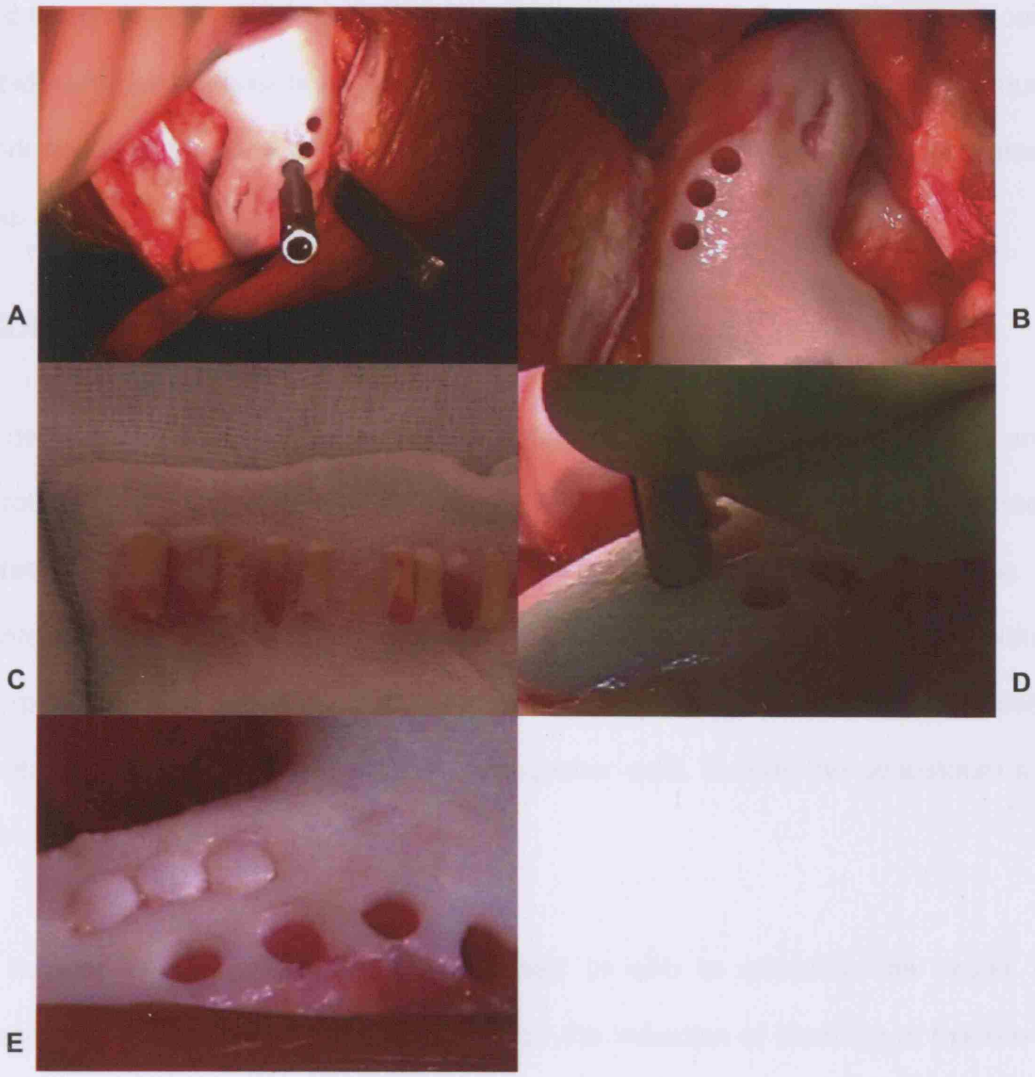



**Figure 2.4** Photographs of the technique of Mosaicplasty. (A&B) Grafts harvested from the harvest site on the medical border of the medial femoral condyle (note the 2x1cm full thickness defect of the medical femoral condyle (the donor site). (C) The osteochondral plugs layed out on to a saline soaked swab (D) The grafts are placed into the donor site (E) the final result (the harvest site defects are left to alone to fill with fibrocartilage).

The PVA is infiltrated onto a titanium fibre mesh to allow it to be attached to bone and the composite was inserted into the femoral condyles of dogs. The composite rapidly attached to host bone and may hold some promise for the future, but much work needs to be done to prove its effectiveness as a surface material for human use.

### 2.2.4 Marrow Stimulation Techniques

Bone marrow contains a multipotential stromal cell known as colony-forming unit-fibroblastic (CFU-F), which is capable of differentiating into chondrocytes, osteoblasts, fibroblasts and adipocytes (Beresford, 1989). Differentiation is controlled by various environmental cues including growth factors, related morphogens and mechanical factors. Other sources of these progenitor cells, hereafter referred to as mesenchymal progenitor cells, include the periosteum and perichondrium (Caplan, 1991).

A number of techniques have been used *in vivo* to stimulate the repair by mesenchymal progenitor cells often through the induction of bleeding at the site of repair. Following the initial damage and bleeding into the defect, a fibrin clot will form containing a small proportion of mesenchymal progenitor cells. These cells then proliferate and differentiate into chondrocytes. In most cases, however, a fibrocartilaginous repair tissue ensues. This tissue consists of type I collagen fibres, in contrast to articular cartilage which contains predominantly type II collagen. The repair tissue, therefore, lacks the normal resilient characteristics of articular cartilage with poor load-bearing properties (Shahgaldi, 1998) and is thus more susceptible to medium to long-term wear. For this reason these techniques are often combined with other surgical procedures such as an osteotomy, to reduce the weight bearing from the affected area of the joint.

**Debridement and Subchondral Drilling**

In a seminal study, Pridie drilled a series of holes into the subchondral bone in an attempt to induce fibrocartilaginous repair tissue in knees that he was debriding for osteoarthritis (later to be reported by Insall (Insall, 1967)). His results were encouraging. However, because the drilling was used in combination with many other procedures such as synovectomy and trimming of osteophytes, it is difficult to differentiate the benefit attributed to the drilling alone.

This procedure was expanded by excising the cartilage and subchondral bone of 85 patients with diseased patellae to expose the cancellous bony bed in a procedure termed "spongialization" (Ficat et al., 1979). They reported good or excellent results in 79% with an average follow up of 15 months.

A further study attempted to replicate Pridie's observations, using histological evidence from an animal model (Meachim and Roberts, 1971). They performed subchondral drilling to full thickness defects in the knees of 21 adult male rabbits. Two years after the procedure, however, the defects had not fully healed and failed to show consistent covering of the defects, even with fibrous tissue.

In a subsequent study 65 articular cartilage lesions were treated by subchondral drilling (Dzioba, 1988). At two years 69% had good results. He found that the group with the best prognosis were those with small to medium sized acute partial thickness lesions on the weight bearing surfaces of the femoral condyles. 95% of these patients had good results.

The long term results of marrow stimulation techniques are not well reported. In a randomized, controlled trial of debridement compared with arthroscopic lavage

alone, Hubbard reported initial success with debridement at 1 year in 80% (n=40) of patients who had not undergone previous operations. This result decreased, however, to 59% (n=32) at the 5-year evaluation (Tandogan *et al.*, 2004).

**Abrasion Arthroplasty & Microfracture**

Another method used to induce repair is abrasion arthroplasty. Johnson described his experience with arthroscopic abrasion arthroplasty reporting that removal of the sclerotic dead bone on the surface exposed small cortical vessels which led to repair (Johnson, 1986). Superficial abrasion was considered to impart a more positive effect on the tissue response than drilling. Indeed, about 50% of the patient radiographs demonstrated a widened joint space after the procedure. This radiographic improvement was even more evident in cases that displayed bone on bone contact on the preoperative x-rays. This was presumably because of formation of new fibrocartilaginous repair tissue, although the amount of new repair tissue did not necessarily correlate with an improvement of symptoms.

Abrasion arthroplasty was also shown to offer short term benefit with an average follow up of one year in 60% of 100 adult human knees with full thickness cartilage defects (Friedman *et al.*, 1984). Again, the results appeared better in younger patients. Other authors however, have failed to show such a good response with abrasion arthroplasty (Baumgaertner *et al.*, 1990; Rand, 1991) with reported success rates of less than 40%.

Ogilvie-Harris and Fitsialos reported the outcome of 441 of 551 patients, who underwent arthroscopic procedures for degenerative arthritis of the knee at 2-8 years follow up. They performed debridement for grade I and II lesions and abrasion arthroplasty for grade III and IV lesions. They found that 68% of the patients had symptomatic relief for at least 2 years, whereas only 53% maintained

this status at 4 years after treatment (Hubbard, 1996). Similarly, other studies involving this approach indicated deterioration with time and unpredictable results (Bert and Maschka, 1989). In one patient a very satisfactory appearance of repair tissue corresponded to a poor functional outcome and a knee replacement was required. This was similar to a case reported by Pridie (Insall, 1967) where a complete cartilaginous repair failed to improve symptoms and required a fusion. This is illustrated in Figure 2.5.

A recent study which examined failed abrasion treatment (n=12) reported a mean time to failure of 21 months. Examination of the repair tissue revealed soft, fibrous, spongiform fibrillated tissue, frequently with central degeneration (Nehrer *et al.*, 1999).

In experimental defects created in rabbits, a comparison of drilling versus abrasion techniques showed that neither predictably restored the articular surface, although the former appeared to produce better long term results (Frenkel *et al.*, 1994).

Steadman popularised the technique of inducing "micro-fractures" using small picks to penetrate the subchondral bone (Steadman *et al.*, 1998). This approach was considered to produce minimal heat necrosis, yet a healing response ensues. No comparative data with for example, drilling, is as yet available. Steadman presented case study data on 72 patients (75 knees) where all patients completed self-administered questionnaires preoperatively and postoperatively. Over the 7- to 17-year follow-up period (average, 11.3 years), patients aged less than 45 who underwent the microfracture procedure for full-thickness chondral defects, without associated meniscus or ligament pathology, showed statistically significant improvement in function and indicated that they had less pain (Steadman *et al.*, 2003).

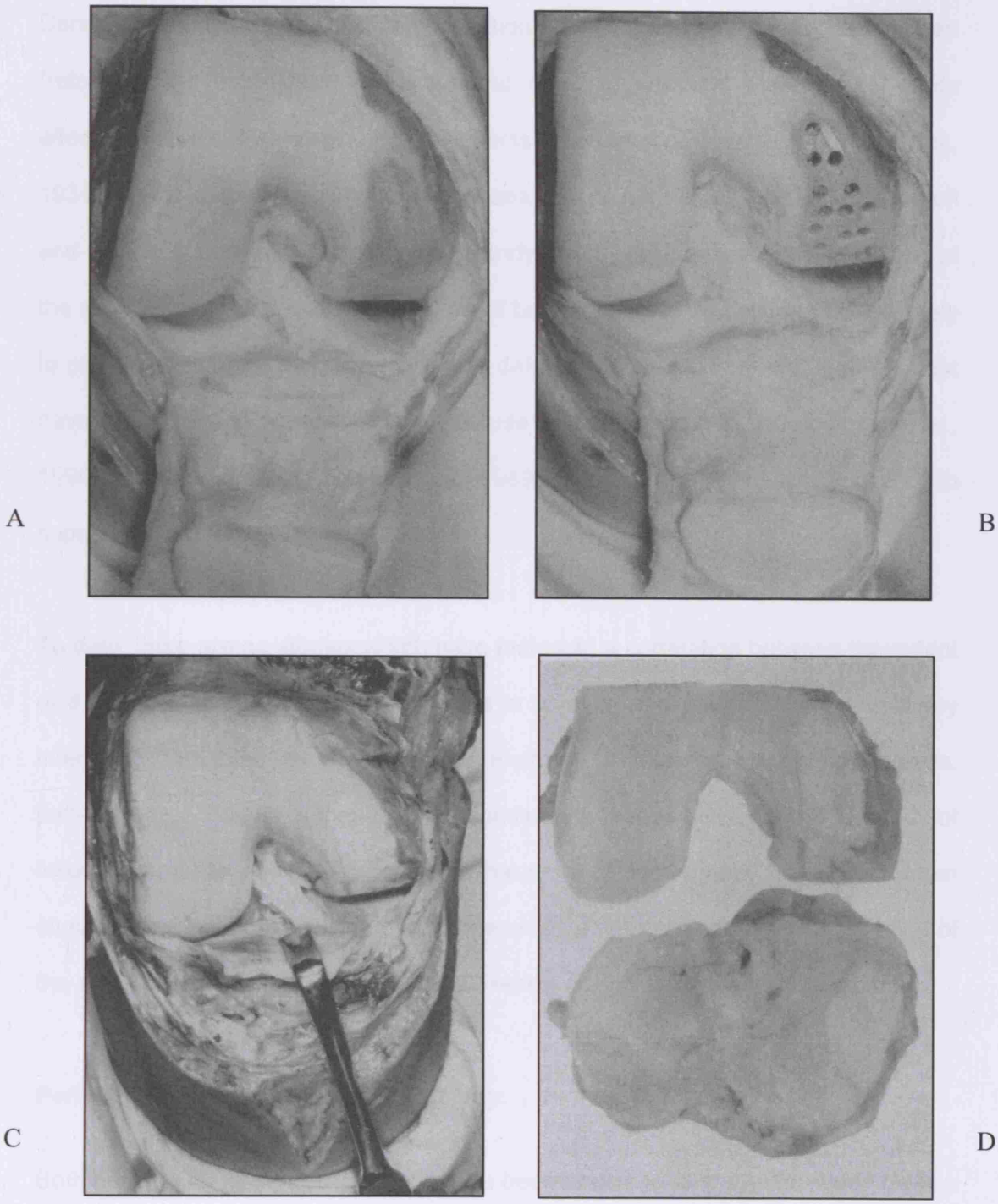



**Figure 2.5** Photographs of a human knee at (A) arthrotomy for degenerative changes to the knee (B) following drilling by Pridie (C) After 15 months at arthrotomy for debilitating pain and (D) resection specimens following fusion of the knee (adapted from "Intra-articular surgery for degenerative arthritis of the knee", Insall 1967).

Dandy stated that the claims for abrasion chondroplasty were unsubstantiated (referring to it as "*miracle surgery*") and described several explanations of its effects; (a) patient selection, (b) the effects of arthroscopy alone (Burman *et al.*, 1934), (c) the associated debridement (Insall, 1967), (d) the period of immobilisation and (e) the charisma of the surgeon (Dandy, 1986). It appears that penetration of the subchondral bone may therefore be of benefit in small defects, but is less likely to produce a long lasting repair in larger defects. Other experimental methods that have been tried to stimulate repair include electrical stimulation (Lippiello *et al.*, 1990) and laser therapy (Hardie *et al.*, 1989). There is little evidence, however, to support their use.

To date, there are no studies which have indicated a correlation between the extent of the repair tissue and an improvement in function and pain. Because so many inter-study variables are involved, for example, animal versus human models, patient age, technique, absence of randomised controlled trials, different periods of follow up and placebo effect, it is clear that proof of such a relationship may prove elusive. Ultimately the procedures provide an unpredictable attempt at restoration of the articular surface, which may not necessarily relieve the patient's symptoms.

**Periosteal & Perichondrial Arthroplasty**

Both periosteum and perichondrium have been shown *in vitro* and *in vivo* to have a chondrogenic potential that permits it to be used for cartilage regeneration (Hall, Jacobson, 1975; Rubak, 1982; Skoog *et al.*, 1972). Indeed in the clinical situation periosteum has been harvested from the proximal medial aspect of the tibia and either sutured (Hoikka *et al.*, 1990; Korkala and Kuokkanen, 1991) or glued (Kreder *et al.*, 1994) to the bed of the osteochondral defect. The chondrogenic potential of free autogenous periosteal grafts was demonstrated in artificially created defects in

the knees of six month old rabbits (Rubak, 1982). By using a cell filter between the subchondral bone and the periosteum the author confirmed the origin of the cartilaginous tissue to the periosteum.

It has also been reported that grafted osteochondral defects under the influence of continuous passive motion healed with predominantly hyaline cartilage containing more than 90% type II collagen and normal water and proteoglycan content (O'Driscoll *et al.,* 1986). This cartilage was able to withstand load bearing without marked deterioration over a twelve month period (Messner and Gillquist, 1993). In control groups, very little repair was apparent.

A further study demonstrated the use of periosteal grafts to reconstruct the articular surface of the patella in thirteen patients (Hoikka *et al.*, 1990). The initial 7 patients had the graft sutured to the margin of the defect, while for the remaining six patients the graft was held using fibrin glue. Classifying according to function and pain, they attained a good result in 8 patients and a fair result in four at an average of four years follow up. One 55-year-old patient with severe patellofemoral osteoarthritis continued to have disabling pain.

The orientation of the graft has proved a matter of controversy. Most authors place the periosteum in such a way that the cambium layer is facing into the defect and the fibrous layer faces into the joint (Hoikka *et al.*, 1990; Korkala and Kuokkanen, 1991), whereas O'Driscoll places the cambium layer upwards facing into the joint. The latter justified this approach during his rabbit experiments by demonstrating worse results in the group in which the periosteum was reversed to face into the defect (O'Driscoll *et al.,* 1986). Other studies have also demonstrated effective repair with the cambium layer facing into the joint (Kreder *et al.*, 1994; Moran and Kim, 1992).

Perichondrial arthroplasty was described by Skoog *et al* in 1972 for the resurfacing of joints of the hand (Skoog *et al.*, 1972). It has been suggested that the transplant of perichondrium produced tissue that was morphologically, biochemically and biomechanically similar to articular cartilage (Woo *et al.*, 1987). In this technique an incision was made over the lowest left side of the chest and the perichondrium was dissected from one of the lower ribs. The graft was invariably held using fibrin glue (Bouwmeester *et al.*, 1997; Homminga *et al.*, 1990) with the chondral side facing the joint.

A further study reports the results of thirty osteochondral defects in the knee, from 25 patients, treated by perichondrial grafting, followed by continuous passive motion (Homminga *et al.*, 1990). Patients were evaluated by arthroscopy, biopsy and radiology. Of the 30 grafted cartilage defects, 27 had filled with tissue resembling cartilage. Three biopsies showed the presence of cartilage, however there was disruption of the bone-cartilage junction in two cases. Fourteen of the 25 patients were reviewed after two years with an excellent improvement in the HSS knee score.

Bouwmeester *et al* reported a four year follow up of 88 patients who had undergone rib perichondrial grafts and revealed poor results in 55% of patients. The authors concluded that the poor results may have been related to overgrowth or calcification of the graft or the preoperative presence of osteoarthritis. In addition, this group immobilised the knee postoperatively (Bouwmeester *et al.,* 1997). The same authors later performed a retrospective analysis of autogenous perichondrial grafting versus subchondral drilling 10 years post-surgery and reported no discernable difference between the two groups. A further group compared the use of periosteum with that of perichondrium in a horse model (Vachon *et al.*, 1989).

Significantly better chondrogenesis was observed in free intra-articular periosteal grafts compared to perichondrial grafts.

**Continuous Passive Motion**

The biological concept of continuous passive motion (CPM) of joints was introduced by a Canadian surgeon, Salter, for the postoperative treatment of many types of articular injury. He and his colleagues demonstrated that the healing of articular cartilage was enhanced in adolescent and adult rabbits by the post-operative use of continuous passive motion. In one experiment, 4 one-millimetre drill holes were introduced into the knee of each rabbit (Salter *et al.*, 1980). Four weeks later, they reported healing with predominantly hyaline cartilage in 60% of the forty defects in the adolescent rabbits and 44% of the forty defects in the adult rabbits. In the immobilized or control rabbits, the latter involving free movement in the cage, only 10% or fewer of the defects had this repair tissue. Subsequent studies showed that although CPM enhanced cartilage healing, the effect was much less pronounced in defects larger than 3mm in diameter (O'Driscoll *et al.*, 1986).

Other work has confirmed the beneficial effects of CPM in conjunction with the use of periosteum in repairing articular cartilage defects (Kreder *et al.*, 1994; O'Driscoll *et al.*, 1988). Based on this data many surgeons now use postoperative CPM, although there have been no proper controlled trials carried out to support this clinical practice. The benefit of continuous passive motion must be balanced against the risk of dislodging the repair in the immediate postoperative period.

### 2.2.5 Chondrocyte Transplantation

#### Allogenic Chondrocytes

Techniques for the isolation and culture of articular chondrocytes were developed several decades ago (Manning and Bonner, 1967; Chesterman and Smith, 1968). However, the first successful transplantation of chondrocytes was reported in 1971 (Bentley and Greer, 1971). These authors found that both isolated epiphyseal and articular cartilage chondrocytes were incorporated into defects in rabbit knees without rejection. In their initial experiments, failures were thought to be due to dislodgement from the grafted area. This study was extended in an attempt to increase the strength of the transplant by culturing the cells in the laboratory. After six weeks in culture, the authors were able to expand the amount of cells by 30 times and these cells produced a matrix that was similar to hyaline cartilage and, in particular, was positive for type II collagen (Aston and Bentley, 1982). In further experiments this cultured material and whole plugs of articular cartilage were transplanted into the knees of adult rabbits (Aston and Bentley, 1986). Examination of the grafts at one year showed 84% successful incorporation of the solid plugs and 64% using cultured chondrocytes. Two reasons given for the lower success with cultured chondrocytes were loss of cells from the defect and problems with the method of culture used. However, the description of a cartilage 'bank' opened possibilities for the future treatment of damaged articular cartilage.

More recently, transplanted allogeneic chondrocytes embedded in agarose gel were transplanted into rabbit articular cartilage defects. Defect repair was analysed and at eighteen months, a morphologically stable hyaline-like cartilage was observed in 47% of the defects (Rahfoth *et al.,* 1998).

**Autologous Chondrocytes**

In 1984 a Swedish group presented their work on successful transplantation of autologous chondrocytes into defects made in rabbit patellae, contained under a sutured periosteal flap (Peterson *et al.*, 1984; Brittberg *et al.*, 1989). They reported that the use of chondrocyte autografts significantly improved the reparative capability of articular cartilage. Further experiments were also carried out to assess what percentage of transplanted cells remained within the repair tissue (Grande *et al.*, 1989). Although 95% of the chondrocytes were labelled at implantation, by six weeks only 8% of the cells remained labelled. Their hypothesis was that a proportion of the chondrocytes survived and multiplied to form part of the intrinsic repair tissue. At six weeks 82% of the grafts had filled in with tissue that resembled articular cartilage compared to only 18% of control defects. It should be noted that this study only reported histological findings without immunolocalisation of, for example, type II collagen.

More recently, fluorescent-labeled articular chondrocytes in a goat model were studied. This study's data indicated that implanted cells persisted in the defect for at least fourteen weeks and participated in the integration with the surrounding tissues, and became part of a repair tissue, rich in type-II collagen and sulphated proteoglycans. At eighteen month follow up, however, the tissue quality had deteriorated and integration with the surrounding native cartilage was poor (Dell'Accio *et al.*, 2003).

In 1996 Brittberg published an animal model looking at cartilage repair in four-month-old New Zealand white rabbits (Brittberg *et al.*, 1996). They reported a mean repair of 87% of the total area in patella chondral defects treated with cells injected under a periosteal flap. This was significantly better than the untreated control lesions or lesions that had been covered by a periosteal flap alone. In addition, a

carbon-fibre pad seeded with chondrocytes yielded only a 31% repair of the total defect area. The authors noted that, by one year follow up, the repair tissue was brittle and this may indicate that it could loosen from the defect and degenerate in the long term. They also found that incorporation of the healing tissue to the surrounding cartilage tended to be incomplete. The repair tissue did, however, appear to mature over time. Thus, by 52 weeks, hyaline-like tissue with more pronounced columnarization was evident compared to the earlier specimens at 12 weeks where cluster formation was apparent in a similar manner to that found in osteoarthritic cartilage. Three theories were proposed to explain the process associated with autologous chondrocyte implantation, namely;

1) The implanted cells repopulate the defect and produce matrix with the periosteal patch acting simply as a watertight seal. Growth factors may or may not penetrate this membrane to enter the defect.
2) The growth factors in the periosteum may stimulate division of the cultured chondrocytes.
3) The cultured chondrocytes and the periosteum interact to stimulate chondrocytes in adjacent host cartilage, subchondral bone and in the periosteum to migrate into the defect and elicit a repair.

Autologous Chondrocyte Transplantation (ACT), more commonly known nowadays as Autologous Chondrocytes Implantation (ACI) was first introduced clinically in 1987. The clinical procedure involves two steps (Brittberg *et al.*, 1994b). At the first stage tissue is harvested arthroscopically from the medial femoral condyle. The chondrocytes are then isolated by enzymatic digestion, and the cells grown in monolayer culture to expand in numbers between twenty to fifty times over a period of about 4 weeks.

To determine the amount of cartilage needed to be harvested, biopsy specimens from 1000 patients were studied. The mean weight of the biopsy tissue was 280 mg

(range, 4 to 1700 mg). The mean cell density, in specimens from 500 patients, was 2600 cells/mg of biopsy tissue (Brittberg *et al.*, 2003). At implantation, the authors recommend a cell density of $30 \times 10^6$ cells.mL$^{-1}$ although the data to support the most appropriate implantation seeding density appears to be lacking.

The second stage procedure takes place after about four weeks and involves an arthrotomy, preparation of the defect, harvest of a periosteal flap, fixation of the periosteal flap to the defect, securing a watertight seal with fibrin glue, implanting the chondrocytes, and wound closure as shown in Figures 2.6-2.7.

The first clinical series included twenty-three patients with cartilage defects in the knee, who were followed up for an average of 32 months, range 16-66 months (Brittberg *et al.*, 1994b). The cartilage defects all extended down to, but not through the subchondral bone. Sixteen patients had defects of the femoral condyles and the remainder had defects in the patella cartilage. The defects ranged in size from between 1.6 to 6.5 cm$^2$. Cells were harvested at initial arthroscopy from the upper outer edge of the medial femoral condyle of the same knee. After multiplication of the cells by about tenfold in medium supplemented with the patient's own serum the cells were injected back into the defect under a periosteal flap, which had been obtained from the proximal medial tibia just below the pes anserinus. The periosteum was sutured to the surrounding cartilage with the cambium layer facing into the defect. The post-operative regime involved early active movements of the knee without weight bearing, which was introduced gradually within the next eight weeks. Two years following the procedure 14 of the 16 patients with femoral condylar repairs were graded as either excellent or good on a subjective grading system based on pain, swelling and locking. Arthroscopic assessment at 12 to 46 months revealed a biologically acceptable gross appearance of the repair in each of these patients. In the two unsuccessful patients, arthroscopy revealed severe

central wear of the transplants. Biopsies were taken from 15 of the sixteen patients with femoral transplants. Eleven biopsies revealed an intact articular surface with the histological appearance of "hyaline like" cartilage. Five biopsies were positive for type II collagens. The results for the patella defects however, were disappointing. Of the 7 patella repairs only 2 were graded as excellent or good. Three were graded as fair and 2 as poor, at a mean follow up of 36 months. At arthroscopy performed 19 months following surgery, only 3 of the 7 repairs had an acceptable appearance. All 7 of the patellar repairs were biopsied. However, in only one biopsy was there evidence of hyaline cartilage. The authors suggest that the poorer results obtained in this subset of patients may be attributed to patellar maltracking.

Breinan together with a group in Boston also investigated the use of this technique in a canine model (Breinan *et al.*, 1997). They looked at defects in the trochear groove as opposed to the patella. The autologous cells were expanded in culture by Genzyme Tissue Repair (Boston, Mass, USA) who began a commercial agency to supply autologous cells following the publication of Brittberg's work. Their three groups consisted of control defects, defects covered by periosteum alone and defects filled with autologous cells and covered by periosteum. At twelve or eighteen months following the procedure they were unable to detect a significant difference between any of the groups. They did, however, find that in the two groups in which the periosteum had been used, there were degenerative changes around the repair that appeared to be related to the suturing. The authors concluded several drawbacks to the canine model: 1) a thinner subchondral bone plate than in humans, leading to more frequent bleeding into the defect; 2) the canine periosteum is thicker than the cartilage to be repaired; 3) canine cartilage is only 0.5-1.0 mm thick compared to humans, which is 3-5mm.

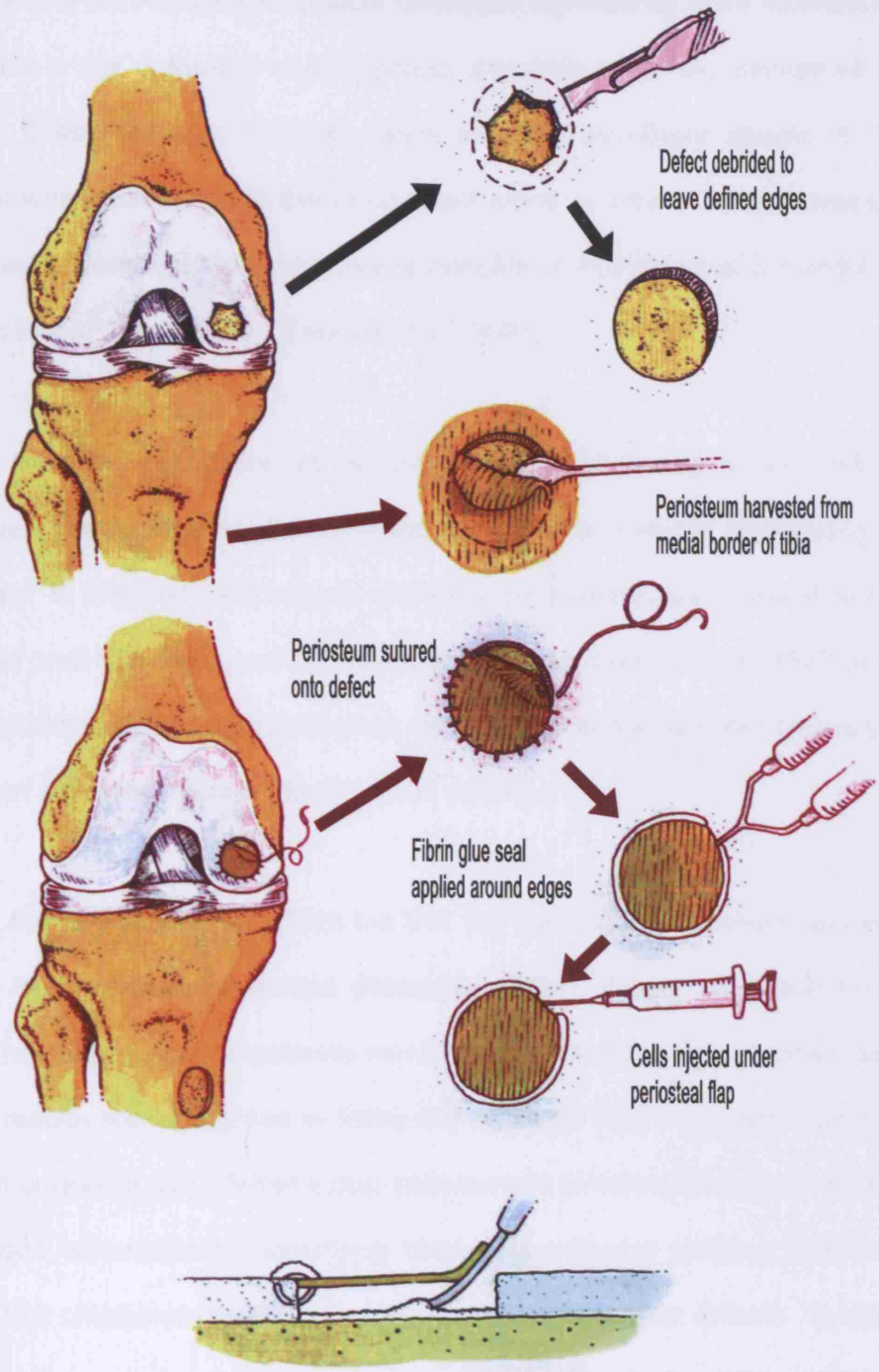



**Figure 2.6** Illustration of the process of Autologous Chondrocyte Implantation (adapted from Brittberg *et al* 2003).

This study was then repeated with a shorter follow up of 1.5, 3, and 6 months and the results were compared to the earlier data in order to assess the change in composition of reparative tissue with time.

After 3 months the ACI-treated defects contained significantly more reparative tissue than found in the untreated control group, including twice the amount of hyaline cartilage. These findings, however, were the only significant effects of the ACI treatment when compared to the periosteum alone or empty control groups. Their findings were therefore, that the positive benefits of ACI found at 3 months did not extend to longer time periods (Breinan *et al.,* 2001).

Despite this, the publication of Brittberg's paper (Brittberg *et al.,* 1994b) and subsequent widespread media coverage led to many patients demanding such a procedure. In Sweden, autologous chondrocyte transplantation combined with a periosteal graft had been used in more than 1200 patients between 1987 and 2003, and, worldwide, variants of autologous chondrocyte transplantation or implantation have been performed in more than 10,000 patients.

In 1998, the long-term results from the first 101 patients, 94 of which had adequate medical records, were presented (Peterson, 1998). Follow up was between 2-9 years. They separated the patients into 5 categories depending on their diagnosis and the results are highlighted in Table 2.2. Overall 77% of patients had either an excellent or good result. Arthroscopic assessment demonstrated good repair tissue filling, good adherence to underlying bone and adjacent cartilage and hardness close to that of adjacent tissue in those with femoral condylar defects. Symptomatic hypertrophic response of the periosteum was identified in 7 arthroscopies. Thirty-seven biopsies were obtained and hyaline-like repair tissue was reported to have correlated with the successful clinical results in 80% of the cases in which biopsies were taken. In seven patients the implant failed within two years of implantation.

To study the long-term durability of autologous chondrocytes transplantation, Brittberg *et al* went on to report the results of the first consecutive 61 patients,

followed up after five to eleven years (mean, 7.4 years). They divided the patients into four groups; Group 1- Isolated femoral condyle lesions (n=19); Group 2 – osteochondritis dissecans lesions (n=14); Group 3 – patellar lesions (n=17) and Group 4 – femoral condyle lesions with ACL reconstruction (n=11).

At two years, 50 of the 61 patients had a good or excellent result, whereas, at the five to eleven-year evaluation, fifty-one of the sixty-one patients had such a result. Four patients needed carbon fibre implants after graft failure, one patient died (of unrelated causes) and one patient did not wish to participate further in the study. Therefore 55 patients were examined at the end of the study. The total failure rate was 16% (ten of sixty-one) at a mean of 7.4 years. All failures of autologous chondrocyte transplantation occurred in the first two years, so a high percentage of the patients who had a good to excellent result at two years had a similar result at the time of long-term follow-up (Peterson *et al.*, 2002).

Knutsen *et al* studied eighty patients who had symptomatic focal cartilage lesions of the femoral condyles measuring 2 to 10 $cm^2$. The patients were treated at four hospitals and were randomized into two groups, namely, those treated with autologous chondrocyte transplantation and those treated with microfracture. They were followed at twelve and twenty-four months. According to the SF-36 physical component score at two years postoperatively, the improvement in the microfracture group was significantly better than that in the ACI group. Both groups had acceptable short-term clinical results (Knutsen *et al.*, 2004).

Bentley *et al* studied 100 patients (mean age, 31.3 years; range, 16 to 49) with symptomatic chondral and osteochondral lesions of the knee that were suitable for cartilage repair. The patients were randomized to undergo either ACT (n=58) or mosaicplasty (n=42). Most lesions were post-traumatic, and the mean size of the

defects was 4.66 $cm^2$. The mean duration of symptoms was 7.2 years, and the patients had a range of 0 to 4 surgical procedures, excluding arthroscopy (mean 1.5). The mean duration of follow-up was nineteen months (range, twelve to twenty-six months). Functional assessment using the modified Cincinnati and Stanmore scores and objective clinical assessment showed the rate of excellent or good results to be 88% after autologous chondrocytes transplantation compared with 69% after mosaicplasty. Arthroscopy at one year demonstrated an excellent or good repair in 82% of the ACT procedures and in 34% of the mosaicplasties. All five patellar mosaicplasties failed. This prospective, randomized clinical trial showed substantial superiority of ACT compared to mosaicplasty (Bentley *et al.*, 2003). The results of the mosaicplasty group were, however, confounded by a few patients who were randomised to the mosaicplasty group, who had large kissing lesions of the patella and femoral or tibial condyles and this group did very badly due to catching of the grafts on movement of the knee.

In contrast Horas *et al* performed a prospective randomized clinical study of ACI versus mosaicplasty and showed very little difference between the groups at two years follow up, although the improvement provided by ACI was delayed compared to that provided by the osteochondral cylinder transplantation. Histologically, the defects treated with ACI were primarily filled with fibrocartilage, whereas the osteochondral cylinder transplants retained their hyaline character, although there was a persistent interface between the transplant and the surrounding original cartilage. The authors acknowledged that their study included only a small number of patients, a relatively short (two-year) follow-up, and no control group (Horas *et al.*, 2003).

Although the Swedish group pioneered this procedure, Genzyme Tissue Repair (Boston, Mass, USA) produce an alternative product, Carticel® which uses specially

selected batches of fetal calf serum rather than autologous serum for the culture. More patients globally have received this product than any other. Genzyme built a data registry reviewed by an Orthopaedic Advisory Board with data from over 580 international sites (440 US, 140 Europe).

**Table 2.2** 2-9 year follow up of ACI. International Cartilage Repair Society (Peterson, 1998).

| Treatment Groups | Patients Evaluated / Total Treated | Brittberg Scoring System Clinical Rating | | | | Patient Overall Assessment | |
|---|---|---|---|---|---|---|---|
| | | Excellent | Good | Fair | Poor | Improved | Same/worse |
| Isolated Femoral Chondyle Defects | 25/25 | 17 | 7 | 1 | 0 | 23 (92%) | 2 (8%) |
| Multiple Lesions | 15/16 | 3 | 6 | 3 | 3 | 10 (67%) | 6 (33%) |
| Osteochondritis Dissecans | 18/18 | 13 | 3 | 1 | 1 | 17 (89%) | 2 (11%) |
| Patella lesions with realignment | 19/19 | 6 | 5 | 6 | 2 | 13 (68%) | 6 (32%) |
| Femoral condyle defects with ACL reconstruction | 16/16 | 7 | 5 | 3 | 1 | 11 (68%) | 4 (32%) |
| TOTAL | 93/94 | 46 | 26 | 14 | 7 | | |

The registry data is now not available publicly although around the time of its inception it was freely published on the Internet. By February 1999 Genzyme had 558 patients at 12 month follow-up, 220 patients at 24 months and 40 patients with 36 months follow-up. In summary at 36 months, 80% of fifteen patients with medial femoral condyle defects improved and 100% of four patients with defects of the lateral femoral condyle improved, as rated by both the patient and physician. No data was supplied regarding patella defects and this data has not been subsequently published in a peer reviewed journal.

**ACI and Associated Knee Injuries**

More often than not, a cartilage injury is associated with other co-existing knee pathologies, such as meniscal injury, biomechanical malalignment or ligamentous insufficiency. These must be dealt with and considered when assessing any results of this technique.

If there is a significant bony defect in addition to the cartilage defect then ACI alone will not provide a sufficient treatment strategy. For example, bone-grafting is recommended for osteochondral defects of more than 8 to 10 mm in depth (Brittberg *et al.*, 2003). This procedure can be done at the time of arthroscopic evaluation and the harvest of the cartilage. Alternatively, a one-stage procedure consisting of autologous chondrocyte transplantation in combination with bone-grafting can be performed. With this so-called "sandwich technique", the bone defect is filled with bone graft, periosteum is sutured on top of the bone graft at the level of the subchondral bone plate, a second layer of periosteum is placed over the cartilage defect, and the chondrocytes are then placed between the layers of periosteum (Brittberg *et al.*, 2003).

**Rehabilitation of ACI Patients**

There seems to be no standardisation of rehabilitation protocol for patients undergoing ACI. Brittberg initiated continuous passive motion on the day after the procedure and a program of gradually increasing weight-bearing was recommended over the subsequent weeks. Beginning in the second week, a progressive closed chain exercise program with light resistance is started. Open chain knee-strengthening is introduced at approximately twelve weeks (Brittberg *et al.*, 2003). Other researchers recommend the application of a full plaster cylinder for

2 weeks postoperatively with immediate full weight bearing but no flexion or extension, so not to disturb the graft site (Bentley *et al.*, 2003; Haddo *et al.*, 2004). No randomized studies to assess the effect different rehabilitation protocols have been published. This introduces yet another confounding factor.

The authors of a systematic review of the effectiveness of ACI suggested that no definite conclusions can be drawn about the clinical effectiveness of the procedure and that it should still be regarded as an experimental procedure (Jobanputra *et al.*, 2001).

**Complications of ACI**

Most of the reported complications are those normally seen with arthrotomies; they include postoperative stiffness, venous thromboembolism, and postoperative infection.

Complications directly related to the graft are uncommon. One specific complication, periosteal hypertrophy, has been reported to present at between seven to nine months postoperatively. Patients complain of "catching" and localized pain, and arthroscopy may reveal substantial hypertrophy. In Peterson's series 28% of 94 patients had periosteal hypertrophy of which a third were symptomatic. Detachment or delamination of the repair tissue has also occurred and graft failure was reported in 7% of Peterson's series (four of the first 23 and three of the next 78 patients) (Peterson *et al.*, 2000).

Henderson *et al* reviewed 22 patients from a total of 135 treated by ACI who had undergone further surgery for pain and symptoms in the knee after a mean of 10.5 months. Of their 31 grafted lesions 77% had lifting 9.7% had detachment of the

periosteal patches for which arthroscopic shaving was performed (Henderson *et al.*, 2004).

As with many surgical procedures, innovation and advancement is often led by a commercial drive and this has been seen with regards to ACI. Before sufficient data was available to prove the long term efficacy of the technique, modifications have been introduced that further confounds the issue.

**Modifications to the ACI technique**

Verigen Transplantation Services (Leverkusen, Germany) modified the Brittberg technique by using a resorbable membrane called the Chondro-Gide which is a type-I/type-III collagen bilayer membrane (licensed from Geistlich Biomaterials, Pharma AG, Wolhausen) instead of a periosteal patch, as illustrated in Figures 2.7 and 2.8. Clinical data has shown similar results to that found by Brittberg (Bentley *et al.*, 2003). Haddo *et al* reported the outcome of a series of 31 patients who underwent ACI using the Chondro-gide® collagen patch. With short term follow up of up to 2 years, the use of Chondro-gide® membrane produced satisfactory results and suggested that there may be a lower incidence of graft hypertrophy as compared to periosteum (Haddo *et al.*, 2004). Examination of the histology at one year of the tissue of 14 patients generated using the Chondro-gide® and reported that 6 patients had the appearance of hyaline cartilage (Briggs *et al.*, 2003). Bentley *et al* took 19 biopsies at one year in their series of 58 patients undergoing ACI using the Chondro-gide® and found the appearance of hyaline cartilage in seven patients (Bentley *et al.*, 2003).

In the late 1990's a new development and modification to ACI was introduced called Matrix-induced Autologous Chondrocyte Implantation (MACI®). In this procedure the cells are cultured on the Chondro-gide® prior to implantation and the graft is

then attached to the chondral defect using fibrin glue rather than sutures. Theoretically, this means the procedure could be performed arthroscopically (Ronga *et al.*, 2004), hence avoiding the need for an arthrotomy. It should be borne in mind however, that at the time this modified procedure was developed no long term studies on the outcomes of its predecessor (ACI using the Chondro-gide®) had been published.

Cherubino *et al* reported the first series of patients undergoing MACI®. Between December 1999 to January 2001, 13 patients ranging from 18 to 49 years (mean age, 35 years) underwent this technique. The mean defect size was 3.5 $cm^2$ (range, 2.0-4.5 $cm^2$). Follow up was very short and only six patients had follow-up of at least 6 months. The data however, was supportive of the new technique and no complications were reported (Cherubino *et al.*, 2003).

MRI was used to evaluate 27 patients undergoing MACI® as compared to microfracture (n=7) (Bachmann *et al.*, 2004). The results suggested that MACI® was superior to microfracture in terms of filling of the defect with regenerating tissue. In a case study of a 25 year old male patient who underwent this procedure arthroscopically for a $2cm^2$ defect, a good short term result was reported (Ronga *et al.*, 2004).

Other versions on the market include the BioSeed®-C (BioTissue Technologies GmbH, Freiburg, Germany) Chondrocytes cultured in autologous serum and then placed into a fibrin gel are then embedded in a 2-mm-thick biodegradable polymer fleece. At arthroscopic surgery, the defect is measured using a scaled needle that is inserted percutaneously, and the fleece is cut to the appropriate size. The damaged area is cleared, the edges are smoothed, and the fleece is attached with bioresorbable sutures at each corner, after which the knee is mobilized to confirm

secure placement. As of May 2003, 70 patients had received these grafts although data on results have not yet been reported.

Impressive short-term results have also been reported with the hyaluronan-based biodegradable polymer scaffold HYAFF-11 (Fida Advanced Biopolymers, Abano Terme, Italy) (Grigolo *et al.*, 2002). The Swedish group led by Brittberg have used a polyhydroxybutyrate patch previously used in cardiac surgery (Brittberg *et al.*, 2003) although no data has been yet published.

Ethisorb (Ethicon, Norderstedt, Germany), a scaffold comprising polyglactin 910/ poly-p-dioxanone, previously used for neurosurgical applications, has also been explored through experimentation in sheep trochlear defects. The cartilage defects were repaired, but many of the resulting biopsies showed vascular ingrowth in the defects, even though the bone space was not opened and the repairs involved only femoral condyle lesions (Brittberg *et al.*, 2003).

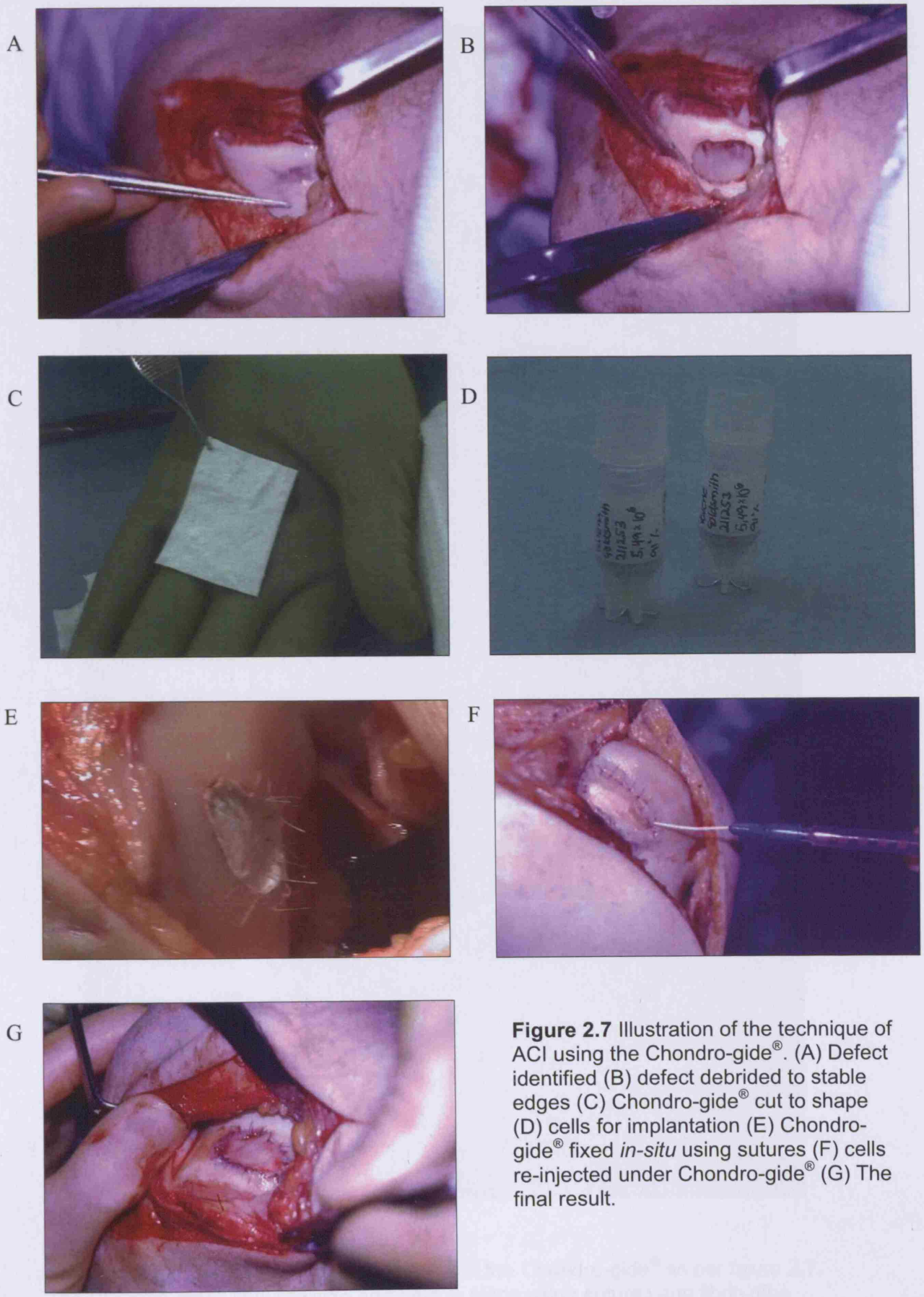



**Figure 2.7** Illustration of the technique of ACI using the Chondro-gide®. (A) Defect identified (B) defect debrided to stable edges (C) Chondro-gide® cut to shape (D) cells for implantation (E) Chondro-gide® fixed *in-situ* using sutures (F) cells re-injected under Chondro-gide® (G) The final result.

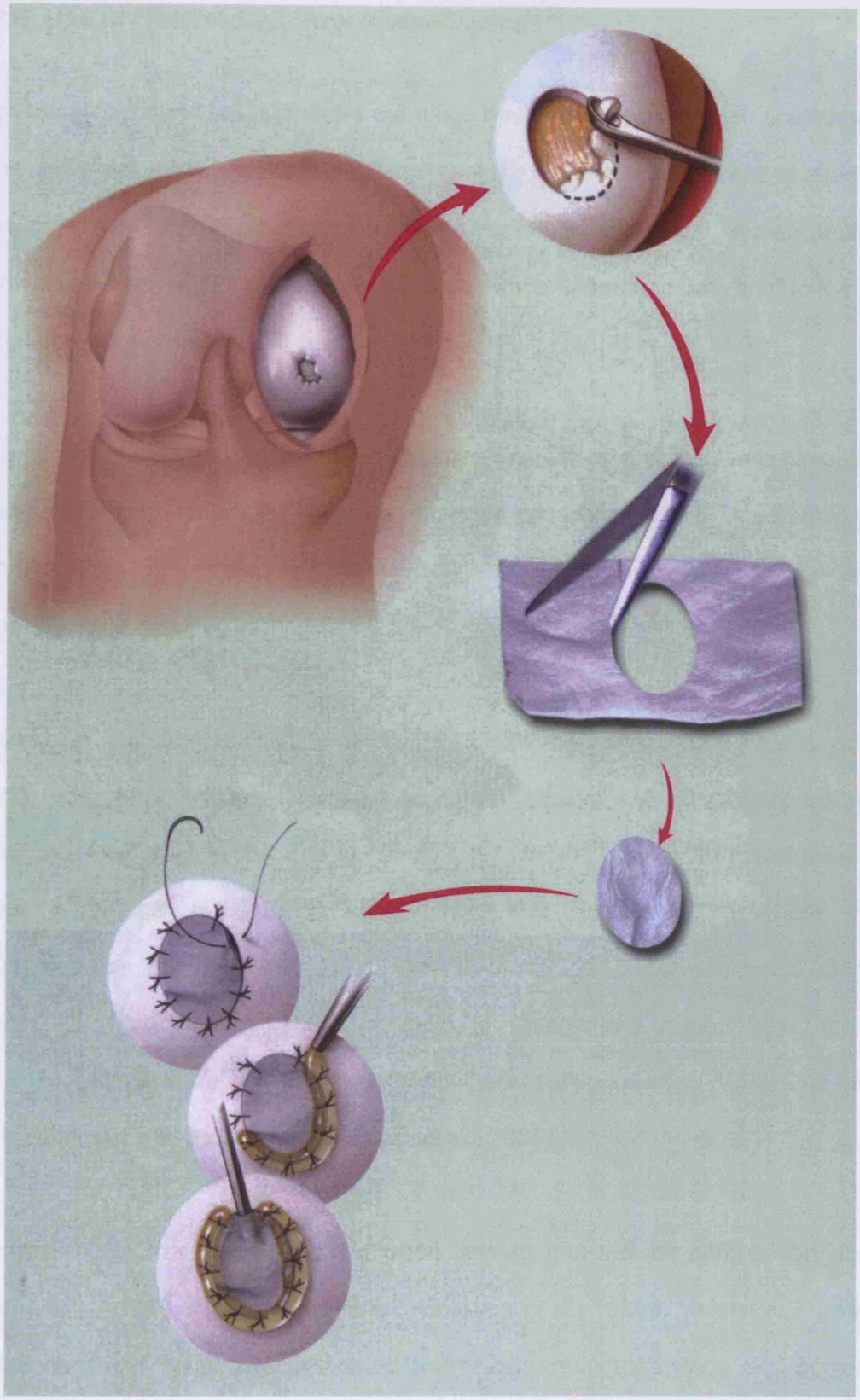


**Figure 2.8** Schematic illustration of the use of the Chondro-gide® as per figure 2.7. The Chondro-gide® is cut to size and held in place using sutures and fibrin glue (illustration courtesy of Verigen UK).

### 2.2.6 Use of Scaffolds/Composites/Matrices

A plethora of natural and synthetic materials have been employed as scaffolds for implantation into articular cartilage defects, alone, or seeded with cells to allow in-growth of new tissue. In addition, these scaffolds will allow growth factors, cytokines, genes, and/or gene products to be concentrated and delivered to the site of repair (Sellers *et al.,* 1997; Andrades *et al.,* 1997; Nehrer *et al.*, 1998b).

In experimental work matrices for engineering articular cartilage *in vitro* have been designed for subsequent implantation into cartilage defects. Both absorbable and non-absorbable materials have been used, as extensively reviewed in a recent paper (Frenkel and Di Cesare, 2004).

Resorbable substances include fibrin (Hendrickson *et al.*, 1994; Paletta *et al.*, 1992), collagen gels (Kawamura *et al.*, 1998; Wakitani *et al.*, 1989), collagen sponges (Nixon *et al.,* 1993; Frenkel *et al.*, 1997; Nehrer *et al.*, 1998b; Nehrer *et al.*, 1998a; Ben-Yishay *et al.,* 1995; Fujisato *et al.*, 1996; Speer *et al.*, 1979; Lee *et al.*, 2003), agarose (Weisser *et al.*, 2001; Rahfoth *et al.*, 1998), hyaluronic-acid (Grigolo *et al.*, 2002; Pavesio *et al.*, 2003), polyglycolic (Vacanti and Upton, 1994; Freed *et al.*, 1993; Sittinger *et al.,* 1996) and polylactic acid (Sittinger *et al.*, 1996; Chu *et al.,* 1995) and bacterial cellulose (Svensson *et al.*, 2005).

Non-resorbable materials that have been investigated include carbon fibre mesh (Minns *et al.*, 1982; Pongor *et al.*, 1992; Brittberg *et al.*, 1994a; Meister *et al.*, 1998) and sponge-like constructs fabricated from polytetrafluoroethylene and polyester (Messner and Gillquist, 1993).

An important design feature is that the material be mechanically stable, even when cut to size by the surgeon to fit the defect. Ideally it should be able to stay in the defect without the need to suture to surrounding cartilage and should be resorbable. It should also maintain chondrocyte phenotype demonstrated by a mechanical integrity to withstand the forces it will be subjected to during normal joint activities and possess a pore structure that accommodates cell infiltration and encourages matrix formation. To date, such a material has yet to been found, but it is likely in the future to play a fundamental role in the tissue-engineering field.

Two related studies repeated the experiments of healing chondral defects over a 15 week period, this time using autologous chondrocyte-seeded type II collagen scaffolds that had been cultured *in vitro* for four weeks prior to implantation (Breinan *et al.*, 1997; Breinan *et al.*, 1998). Although they found good filling of reparative tissue, with less fibrous tissue than previous controls, they also showed a 20-fold lower stiffness of the repair tissue on indentation testing compared to native articular cartilage (Lee *et al.*, 2003).

Collagen gels embedded with allografted chondrocytes have also been used to repair chondral defects (Wakitani *et al.*, 1989). They reported what they referred to as "complete healing" at four weeks in 7 of the 9 treated defects compared to "no healing" in the controls.

Svensson *et al* used bovine and human chondrocytes to compare bacterial cellulose with tissue culture plastic and calcium alginate (Svensson *et al.*, 2005). They found unmodified bacterial cellulose showed significantly higher levels of chondrocyte growth, using TEM analysis and RNA expression of the collagen type-II. Most of the current published work in this area is however, based on animal experiments and therefore, in the early stages of development.

Hung *et al* investigated the use of a bilayered scaffold of chondrocyte-seeded agarose on natural trabecular bone in an attempt to develop an anatomically shaped osteochondral constructs for clinical use (Hung *et al.*, 2003).

In terms of human data, Pavesio et al implanted over 600 hyaluron-based scaffolds (Hyalograft C®) containing autologous chondrocytes into the knees of patients without periosteal coverage. Arthroscopic and histological results from a cohort of 67 patients followed up for a mean of 17.5 months revealed 97% of the patients had subjective improvement postoperatively, and 94% reported a better quality of life. Objectively, knee function was reportedly improved in 87% of the patients, and arthroscopic assessment revealed biologically acceptable results in 97%. Hyaline-like cartilage was seen histologically (Pavesio *et al.,* 2003). It should be stated that this study was industry sponsored.

This detailed analysis of the literature, in the area of cell based repair of articular cartilage defects, reveals a promising future for patients currently debilitated with articular cartilage injuries. However, there are many methodological inadequacies and inconsistencies and moreover there is a paucity of information to characterise the cells being used in current clinical situations as well as the appropriate stimuli to induce optimal cartilaginous repair. It might be concluded that such studies would be better performed in vitro using basic science approaches, involving established model systems to guarantee reproducible experimental conditions.

## 2.3 Aims & Objectives

The overall aim of this study is to investigate, in vitro, the suitability of a variety of sources of cells for cell-based cartilage repair.

This has been achieved through the following set of focussed objectives:

1) To determine the effects of TGFβ on the re-expression of a chondrocytic phenotype and their subsequent ability to produce cartilaginous ECM, using human chondrocytes used in clinical practice for ACI.

2) To investigate the potential of harvesting cells from either the defect site or the patient's bone marrow, as a replacement for the cells currently harvested from a healthy non load bearing area of the affected joint.

3) To determine the effect of in vitro mechanical loading on cells for use in cell based cartilage repair.

# CHAPTER THREE–CHEMICAL STIMULATION OF CELLS

## 3.1 Introduction

An increasing number of patients worldwide have undergone the procedure of Autologous Chondrocyte Implantation (ACI) for cartilage defects of the knee joint and there is clinical follow up, extending beyond 3 years, for more than 1500 patients. A recent development from the basic ACI procedure, termed Matrix-induced Autologous Chondrocyte Implantation (MACI®) involves in vitro seeding the cells onto a collagen membrane for a few days prior to implantation. The short to medium term success rates for ACI range from 60-90% depending on site and location of the cartilage defect (Brittberg *et al.*, 1994b; Peterson *et al.*, 2000) and a histological appearance of a hyaline-like repair is reported to correlate with successful clinical results in 80% of biopsies (Peterson *et al.*, 2000). However, in some cases the implant failed and only fibrous tissue was subsequently evident in the defect.

It is well established that during *in vitro* monolayer expansion, cells typically dedifferentiate to a fibroblastic morphology and phenotype (Holtzer *et al.*, 1960). This de-differentiation manifests itself as a production of type I collagen (found in fibrocartilage), as opposed to type II collagen (characteristic of hyaline cartilage). Nonetheless, it has been shown that dedifferentiation in monolayer is reversible and the cells may regain their chondrocytic phenotype when cultured in conditions which induce a rounded morphology, such as agarose (Benya and Shaffer, 1982), alginate (van Osch *et al.*, 2001; Bonaventure *et al.*, 1994; van Osch *et al.*, 1998; Yaeger *et al.*, 1997) or pellet culture (Jakob *et al.*, 2001). It has also been reported that re-expression of a differentiated phenotype is enhanced by the presence of defined growth factors, such as TGFβ, insulin or IGF-I (Benya and Shaffer, 1982;

Yaeger *et al.*, 1997; van Osch *et al.*, 2001). Moreover, the culture conditions used for monolayer expansion may influence the subsequent response of the cells to growth factors, known to enhance the re-expression of the chondrogenic phenotype (Yaeger *et al.*, 1997; Benya *et al.*, 1988; Benya and Padilla, 1993; Galera *et al.*, 1992; Jakob *et al.*, 2001).

Despite the emergence of tissue engineered strategies in clinical practice, there remains a paucity of information regarding the influence of growth factors, such as TGFβ, during the re-expression process associated with ACI. Accordingly, in this chapter the author aims to examine:

- Chondrocyte isolation using both bovine and human chondrocytes
- The effects of monolayer culture on these cells

These objectives will effectively test the hypothesis that a TGFβ1-containing defined culture medium enhances the re-expression of a chondrocytic phenotype and the subsequent production of cartilaginous ECM by human chondrocytes used in clinical practice for ACI.

## 3.2 Materials and Methods

### 3.2.1 Cell sources

#### Isolation of Bovine Articular Chondrocytes

The test procedures were similar to those detailed in previous studies (Lee and Bader, 1997). Fresh young bovine front feet (18-month-old steers) were obtained from a local abattoir and were washed in water, with special attention being paid to clean the hoof region. The feet were then placed in a bucket of 70% industrial methylated spirit (IMS) for 2 minutes. Once cleaned, the metacarpo-phalangeal joints were opened under aseptic conditions to expose the joint surfaces. The

procedure was performed within 3 hours of animal sacrifice. Full-depth slices of articular cartilage were scraped off using a sharp size 15 scalpel blade, with attention to avoid removing any of the subchondral bone plate. Slices from at least three separate joints were pooled for each experiment. The pieces of cartilage were placed in a Petri-dish and were washed briefly in Earle's Balanced Salt Solution (EBSS, Gibco, Paisley, UK) and cultured at 37°C/5% $CO_2$ for 16 hours in Dulbecco's minimal essential medium supplemented with 10% (v/v) fetal calf serum (DMEM+10% FCS, Gibco, Paisley, UK).

The pieces of cartilage were then chopped into smaller pieces (approximately 2mm x 2mm) to improve enzymatic digestion and incubated at 37°C for 1 hour in 10mL of DMEM + 10%FCS + 700unit.mL$^{-1}$ pronase (BDH Ltd, Poole, UK). The supernatant was removed and replaced with 20mL of DMEM+10%FCS+100unit.mL$^{-1}$ collagenase type Ia (Sigma, Poole, UK) and incubated for 16 hours overnight at 37°C on a rolamixer.

The supernatant containing the released chondrocytes was passed through a 70μm pore size cell sieve (Marathon Laboratory Supplies, London, UK) and the filtrate was centrifuged at 2000 x g for 7 minutes. The supernatant was aspirated and the cells were washed by two further resuspensions in 10mL DMEM+10%FCS. A cell count was performed using the Trypan blue dye exclusion test (Fong and Kissmeyer-Nielsen, 1972) and the cells were plated in tissue culture flasks at an initial seeding density of 1 x $10^4$ cells.cm$^{-2}$ (175 cm$^2$ growth area, Falcon, Oxford, UK) with 35mL of DMEM+10%FCS. The cells were maintained at 37°C/5% $CO_2$ in DMEM + 10%FCS and the culture media was changed every two days. After 7 days in culture, the chondrocytes were rinsed twice in phosphate buffered saline, pH 7.4 (PBS, Sigma, Poole, UK) and recovered by incubation for 15 min in 0.25% (w/v) trypsin (Sigma, Poole, UK) in PBS. The cells were counted and re-seeded into

flasks as above for further expansion. A total of three passages were performed, at 7 day intervals or when the cells had reached 80% confluence.

**Isolation of Human Autologous Passaged Chondrocytes**

Chondrocytes were isolated from the medial femoral condyles of 24 patients, mean age 34 (range 18-49) undergoing ACI. This procedure involves the harvesting of a piece of cartilage (200-300 mg or approximately a thumb nail in size) from a healthy non load-bearing area of the knee. The cartilage was placed in a nutrient medium and transported by overnight courier to the laboratories of Verigen Transplantation Services, Leverkeusen, Germany. The processing technique was proprietary to Verigen, but involved similar techniques to those described above except that the cartilage was digested and expanded in monolayer culture using medium supplemented with autologous serum, for approximately 4 weeks. Following expansion, the cell number and viability of a proportion of the cells from each patient was assessed at the supply laboratory, using the trypan blue exclusion dye test (Fong and Kissmeyer-Nielsen, 1972). The cells were subsequently couriered by overnight delivery in a proprietary transport medium back to the centre for re-implantation into the patient in a second operative procedure. Within an hour of the operative procedure, cells deemed surplus to the requirements of the ACI procedure, were taken to the laboratory for independent assessment.

**Procedure for counting cells**

This technique involves the use of the trypan blue dye exclusion test (Fong and Kissmeyer-Nielsen, 1972) and a Neubauer counting chamber. A 50μL aliquot of a well mixed cell suspension was placed into a small 1.5mL microfuge tube. To this a known volume of trypan blue (see Appendix A) was added to obtain the final dilution of the cells. A Neubauer counting chamber was prepared by placing a glass

cover slip over the counting area. A small volume of the cell mixture was placed onto the counting platform and the cells were counted under a microscope as indicated in Figure 3.1

**Procedure for trypsinising adherent cells**

When the cells reached confluence, the medium was removed from the flask and the cells washed once with PBS at 4°C. One millilitre trypsin solution (see Appendix A) was added to a flask of 25 $cm^3$ or 2mL to a flask of 75 $cm^3$. The cells were covered and incubated at room temperature for 5 minutes. The cells that had not dislodged were detached from the surface using a cell scraper. Five millilitre DMEM/10%FCS was added to the flask and the suspension was removed and centrifuged at 2000 rpm for 5 minutes. The medium was removed with a pipette, being careful not to disturb the cell pellet. The cells were resuspended in 10mL DMEM/10%FCS, as a further wash, and then re-centrifuged at 2000 x g for 5 minutes. The cells were resuspended in 2mL DMEM/10%FCS and passed through a 23G needle to produce a single cell suspension. The mixture was then made up to 10mL for counting as described above.

**Audit of Chondrocyte Numbers and Viability**

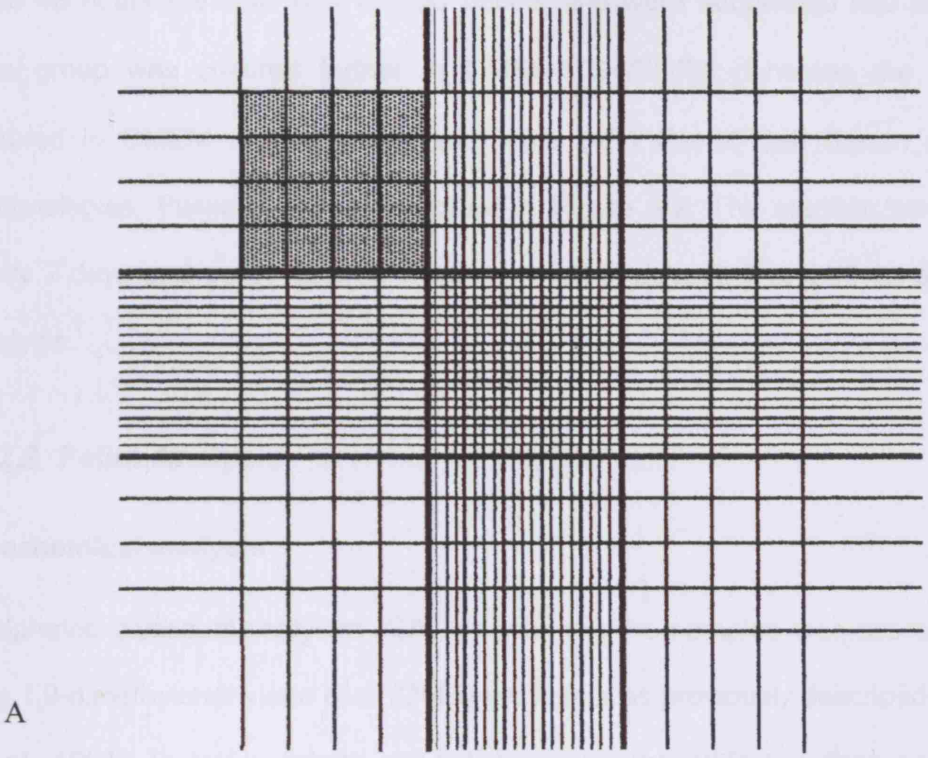
An estimation of cell number and viability was performed on cells from 24 patients. Cells from 4 of these patients, selected in a random manner, were incubated for a further 16 hours at 4°C prior to assessment using the trypan blue method as described above.

**Development of the Pellet Model to Assess Chondrogenesis**

The pellet culture system has been originally described (Holtzer *et al.*, 1960), then adapted (Manning and Bonner, 1967) using human chondrocytes and later described using mesenchymal progenitor cells (Johnstone *et al.*, 1996). As the availability of human chondrocytes for experimentation was limited, bovine chondrocytes were used for all preliminary experiments and this also allowed for a thorough characterisation of the system before use of valuable human cells.

Aliquots containing 4 x $10^5$ cells from ten patients were centrifuged at 500g in 15mL polypropylene conical tubes (Johnstone *et al.*, 1998). The supernatant was removed and replaced with DMEM/10% FCS and the pellets were cultured at 37°C/5%CO2. The medium was changed every 2 days and pellet cultures were removed at days 2, 7 and 14 for subsequent analysis.

For experiments involving human cells it was found that the cells would not pellet initially in DMEM/10%FCS and so the medium was replaced by a defined medium comprising of Dulbecco's Modified Eagles Medium, ITS+ Premix [Collaborative Biomedical Products: insulin (6.25$\mu$g.mL$^{-1}$), transferrin (6.25$\mu$g.mL$^{-1}$), selenous acid (6.25$\mu$g.mL$^{-1}$), bovine serum albumin (1.25mg.mL$^{-1}$), linoleic acid (5.35$\mu$g.mL$^{-1}$)]. Pyruvate (1mM), dexamethasone ($10^{-7}$ M) and 10ng.mL$^{-1}$ TGF$\beta_1$ (recombinant human, R&D Systems) were also added. This medium which has been described previously (Johnstone *et al.*, 1998) is subsequently referred to as DMEM/ITS+/TGF$\beta_1$.



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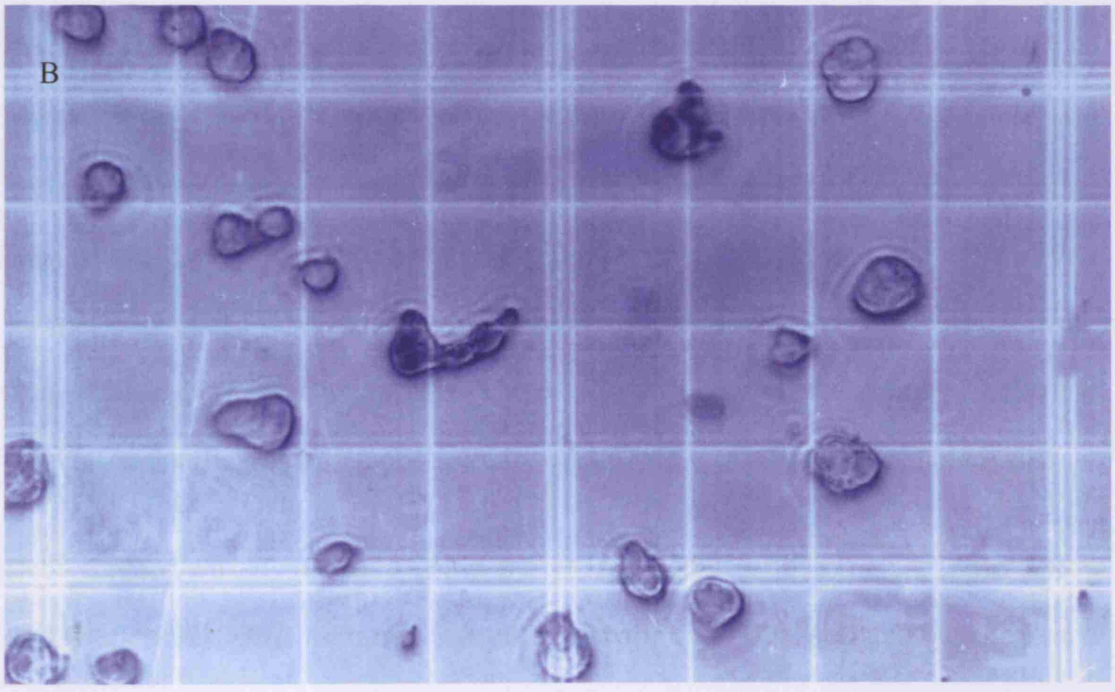


B

**Figure 3.1** Neubauer counting chamber (A) illustrative (B) photograph. Note that the viable cells within the shaded area on (A) and the area diagonally opposite are counted and the mean is calculated (M). The total number of cells (N) in the original suspension (volume V) is calculated by $N=MxDx10^4xV$, where D is the dilution with Trypan Blue.

After 48 hours the cells had formed pellets and were subdivided into two groups. One group was cultured further in DMEM/ITS+/TGF$\beta_1$, whereas the other was cultured in DMEM supplemented with 10% (v/v) Foetal Calf Serum (FCS, Life Technologies, Paisley, UK) as indicated in Figure 3.2. The medium was changed every 2 days and pellet cultures were removed at days 2, 7 and 14 for subsequent analysis.

### 3.2.2 Pellet Analysis

#### Biochemical analysis

Sulphated glycosaminoglycan (GAG) content in the samples was assessed using the 1,9-dimethylmethylene blue (DMB) technique as previously described (Farndale *et al.*, 1982). To review briefly, cell pellets were removed from culture and digested by incubation for one hour at 60°C in 0.5mL papain digest buffer [sodium citrate (55mM), sodium chloride (150mM), cysteine hydrochloride (5mM), EDTA (5mM); all Merck, Poole, UK] and 1$\mu$g.mL$^{-1}$ papain (Sigma, Poole, UK). A standard curve was prepared from chondroitin sulphate A obtained from bovine trachea (Sigma, Poole, UK), using GAG concentrations from 0-50 $\mu$g.mL$^{-1}$ in 5$\mu$g increments. Forty microlitre of both the standards and samples were pipetted in to a 96 well plate. Then 250$\mu$L of DMB solution was added to each well and absorbance at 595 nm was determined using a microplate reader (Bio-Rad Ltd, Hemel Hempstead, UK).

DNA content was determined using the Hoescht 33258 fluorometeric assay (Rao and Otto, 1992). DNA from calf thymus (Sigma, Poole, UK) was used to prepare a standard curve. Using a serial dilution technique, the DNA standard concentrations ranged between 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0 $\mu$g.mL$^{-1}$ in sodium citrate buffer (SSC). The samples were digested in papain digest buffer, as described above and 100$\mu$L aliquots of samples and standards were pipetted into a 96 well

plate. Each standard concentration was used in triplicate. Then 100μL Hoechst (1μg.mL$^{-1}$) was added to each of the wells and the plate was analysed using a microplate fluorimeter (Fluoroskan Ascent, LabSystems, Oxford, UK), with excitation set at 360nm and emission monitored at 460nm.

**Morphology and immunolocalisation**

For histological and immunohistochemical analyses, samples were fixed in 4% paraformaldehyde for 4 hours and then dehydrated sequentially in a series of graded alcohol washes, then in xylene prior to embedding in paraffin. Sections, 5μm in thickness were prepared. The sections were deparaffinised and rehydrated by immersion in sequential Xylene, 100% Alcohol, 70% Alcohol and finally water. For histological analysis the sections were stained with Toluidine Blue (1% in 50% isopropanol) or 1% Safranin-O.

**Immunolocalisation of collagens type I, II and X**

For immunolocalisation, the streptavadin biotin-immunoperoxidase method (Wood and Warnke, 1981) was used to localise collagens type I & II. Briefly, sections were dewaxed in xylene and dehydrated in a series of graded alcohols. Sections were then immersed for 15 minutes in an endogenous peroxidase blocking solution, made up fresh on the day of processing, containing 3mL hydrogen peroxide (100 volume) and 97mL methanol. The sections were then washed in tap water for 10 minutes, during which time a trypsin solution containing 100mL of 0.1% calcium chloride and 100 mg trypsin was made up, at pH 7.8. The sections were immersed in pre-warmed water at 37°C for 10 minutes and transferred into the trypsin also at 37°C in the same water bath. The sections were washed in tap water for a further 10 mins. After rinsing, the sections were left to soak in 0.05M Tris buffered saline + 0.15M sodium chloride at pH 7.6.

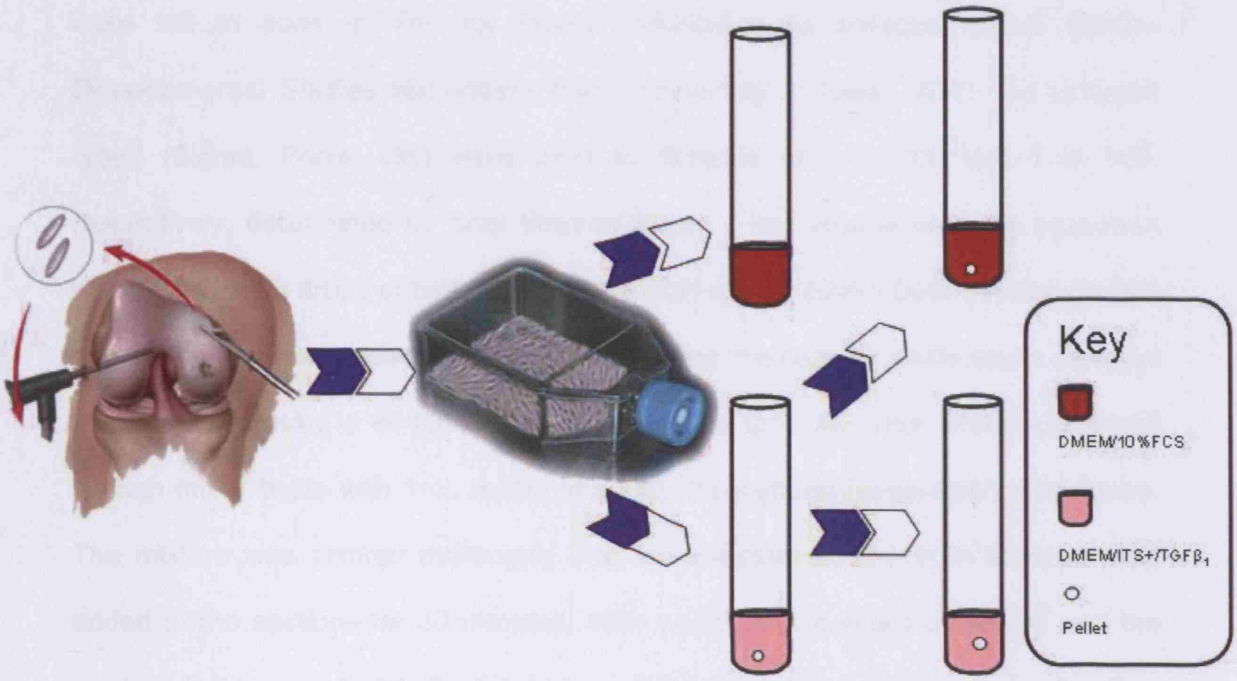



**Figure 3.2.** Schematic describing the experimental procedure for the human autologous passaged chondrocytes. A biopsy was harvested from the medial femoral condyle, the cells are expanded in monolayer and pelleted in either DMEM/10%FCS or DMEM/ITS+/TGFβ1. Note no pellets formed when the pellets were initially cultured in DMEM/10%FCS.

The area around the specimen was wiped dry and 3 drops of normal rabbit serum (Dako X0902, Sigma, Poole, UK) diluted 1:5 in Tris were added to the specimen for 20 minutes, to block non-specific antibody binding sites. The primary antibody was then applied for 60 minutes, after which time it was washed off and the sections were left to soak in Tris for 5mins. Antibodies to collagen type-II (CIICI–Developmental Studies Hybridoma Bank, University of Iowa, USA) and collagen type-I (Sigma, Poole, UK) were used in dilutions of 1 in 100 and 1 in 500, respectively, determined by prior titration assay. The area around the specimen was wiped and 3 drops of biotinylated rabbit anti-mouse (Dako E03554) at a dilution of 1:200 in Tris was added for 30 minutes. During this time an avidin-biotin complex was prepared using a kit (Dako K0377) such that 5mL Tris was added to a small custom mixer bottle with 1mL avidin and 1mL biotinylated horseradish peroxidase. The mixture was shaken thoroughly and the resultant avidin-biotin complex was added to the sections for 30 minutes, after which time it was washed off and the sections left to soak in Tris for 5 minutes. A DAB solution was prepared using Fast DAB peroxidase tablets (Sigma, Poole, UK) by adding one DAB tablet and one urea hydrogen peroxide tablet to 15mL of distilled water. The area around the specimen was wiped and the DAB solution was applied for 10 minutes. The sections were finally washed in running water and counterstained in Harris's haematoxylin for 2 minutes prior to washing in tap water for 5 minutes, differentiating in acid alcohol for 30 seconds, dehydrating, clearing and mounting in DPX. A negative control was prepared in exactly the same manner as described above, except that no primary antibody was added.

**Transmission Electron Microscopy**

Samples for transmission electron microscopy were fixed in 0.2% glutaraldehyde (GA) in 4% paraformaldehyde fixative, in a 0.1M sodium cacodylate buffer at pH 7.2

for 16 hours. After initial fixation the tissue samples were stored in a 0.1M sodium cacodylate buffer containing 0.1M EDTA until processing. The samples were then fixed in 1% osmium tetroxide in 0.1M sodium cacodylate for 60 minutes, washed in 0.1M sodium cacodylate followed by dehydration through a graded series of ethyl alcohols (70%, 80%, 90%, 96%, 100%) and infiltrated with several changes of Spurrs' resin. The specimens were placed into embedding moulds and cured for 18 hours at 70°C. After polymerization, 1µm sections were cut on a LKB Ultratome III ultratome and stained with 1% Toluidine Blue in 1% Borax and viewed under an Olympus BH-2 light microscope. The areas of interest were selected, which included a representative section displaying both cells and matrix. Resin blocks were further trimmed after which 90nm sections for TEM were cut using a LKB Ultratome III ultramicrotome and a diamond knife. Sections were detected on copper grids, stained with 2% uranyl acetate and lead citrate and viewed on a Philips CM12 Electron Microscope at 80 kV.

### 3.2.3 Statistical Analysis

Unless otherwise stated, three separate experiments were performed. At each time point three replicate samples were analysed providing a total of nine replicate values from 3 experiments. Differences between the values obtained for each culture medium were analysed using, an unpaired Student's t-test. Values of $p<0.05$ were considered to indicate a statistical level of significance.

## 3.3 Results

### 3.3.1 Bovine Chondrocytes

During monolayer expansion, the cells adhered to the growth surface of the culture flask and acquired a spindle-shaped fibroblastic appearance as indicated in Figure 3.3, for both days 3 and 14 in culture.

**Biochemical Analysis**

Figure 3.4 illustrates total GAG (Figure 3.4A) and DNA (Figure 3.4B) for pellets containing bovine cells, cultured for up to 14 days in DMEM+10%FCS. DNA levels increased by 67% from day 2 to day 14. There was no statistical significance between values between day 2 and day 7 ($p>0.05$), however, mean DNA values at day 14 were significantly greater than day 7 ($P<0.05$).

GAG levels increased slightly throughout the culture period. There was a small increase of 7% in the day 14 value compared to that on day 2. The difference between day 7 and 2 values were not statistically significant ($p>0.05$), although mean GAG values at day 14 were significantly greater than day 7 ($p<0.05$).

**Pellet Formation**

After about 24 hours the cells started to form an intact structure that lifted off from the bottom of the tube. By 24 hours in culture the pellets had formed disc-shaped constructs that floated in the bottom of the test tube.

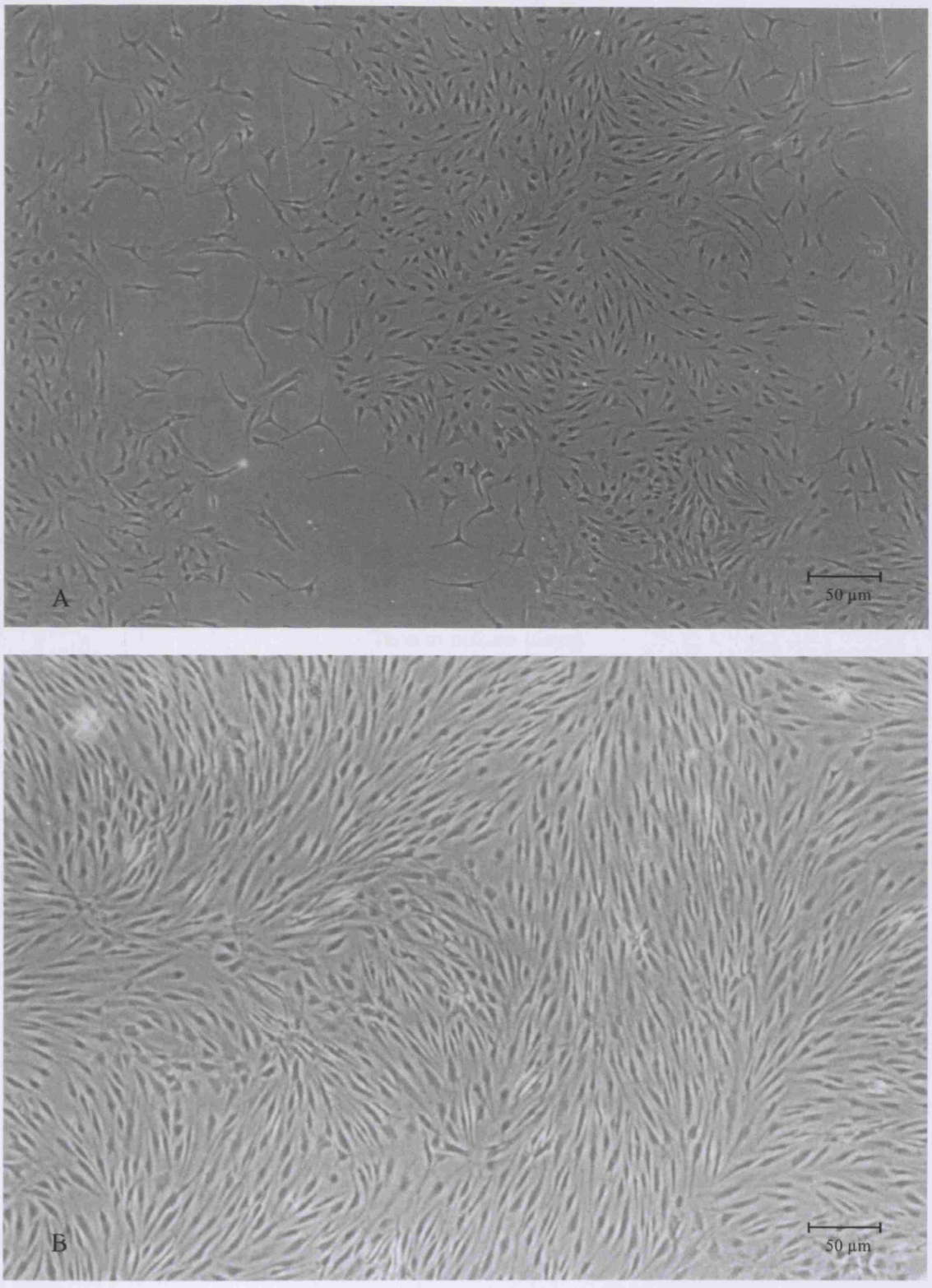



**Figure 3.3** Photograph of bovine chondrocytes cultured in DMEM/10%FCS in monolayer culture (A) after 3 days in culture showing areas of confluence and gap areas, (B) confluence after 14 days in culture.

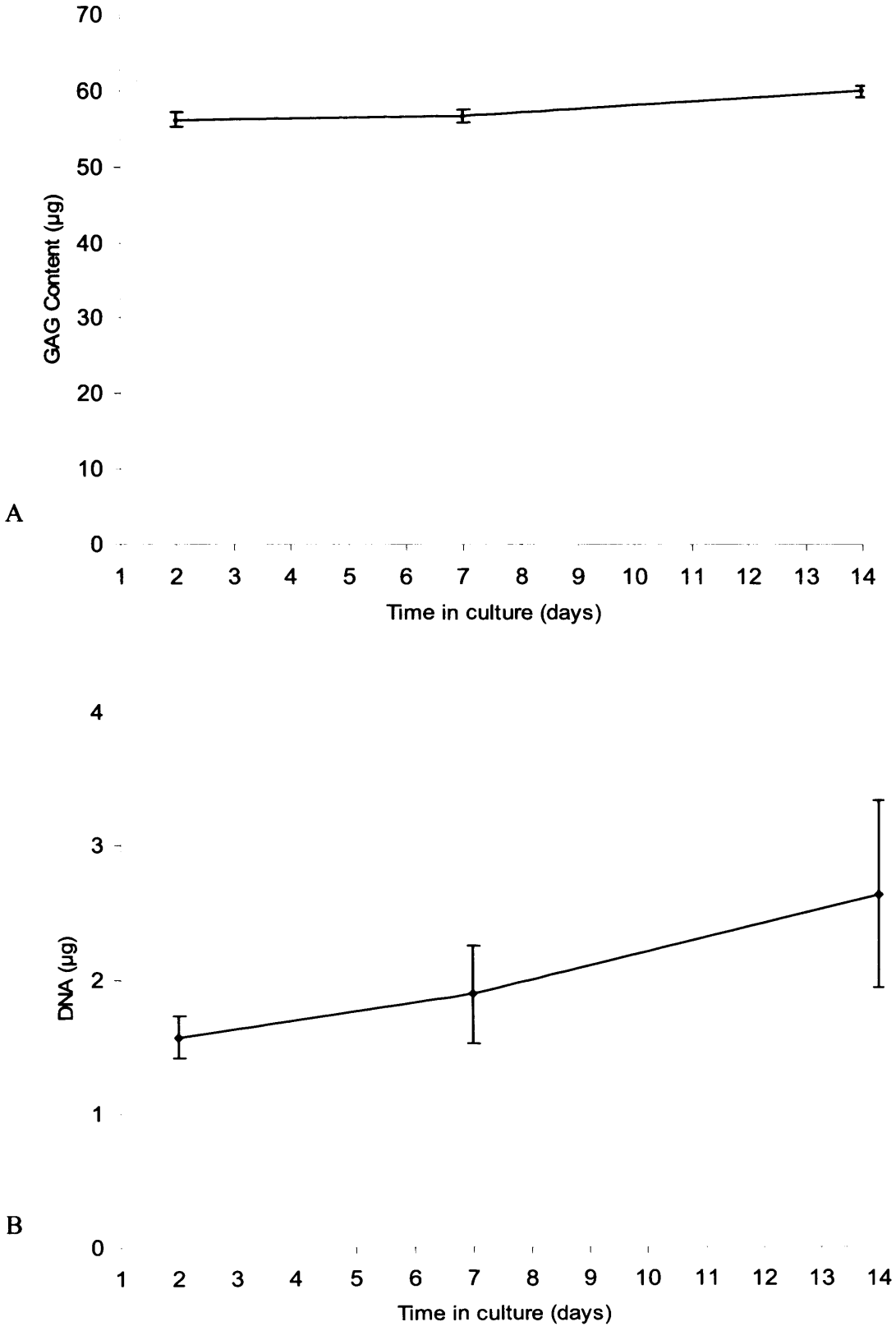



**Figure 3.4** GAG content (A) and DNA content (B) of pellet cultures prepared using bovine monolayer-expanded chondrocytes. The pellets were cultured for up to 14 days in DMEM+10%FCS. Each point represents the mean and standard error of nine replicates from three separate experiments.

**Morphology and immunolocalisation**

Standard histology demonstrated a C-shaped structure, with concentrated cells in the up facing (concave) side and sparsely distributed cells appearing to form vertical columns on the convex surface. There was abundant ECM on the convex (deeper) layer, which stained metachromatically with toluidine blue (Figure 3.5A). There was uniformly distributed staining with safranin O mainly in the region of the abundant matrix (Figure 3.5B). Immunolocalisation revealed extensive labelling for type II collagen throughout the pellets by day 7 (Figure 3.6A), whereas type I staining was indistinguishable from the negative control (Figure 3.6B).

## 3.3.2 Autologous cells

**Cell Number and Viability**

A detailed summary of cell numbers and viability at the supply and test laboratories are listed in Table 3.1. The mean viabilities were 95.79% and 95.56% for the test (n=24) and supply (n=20) laboratories respectively. As indicated in Figure 3.7, these differences were not statistically significant ($p>0.05$). However, for the four batches of cells, which were incubated overnight at 4°C, the corresponding viability assessed at the test laboratory had reduced to 60%. The mean cell numbers were $4.99 \times 10^6$ and $3.74 \times 10^6$ for the test (n=24) and supply (n=24) laboratories, respectively (Table 3.1). These differences were statistically significant ($p<0.001$).

**Pellet Formation**

Rounded pellets had formed in all of the cells cultured in DMEM/ITS+/TGFβ1, within 48 hours as shown in Figure 3.8.

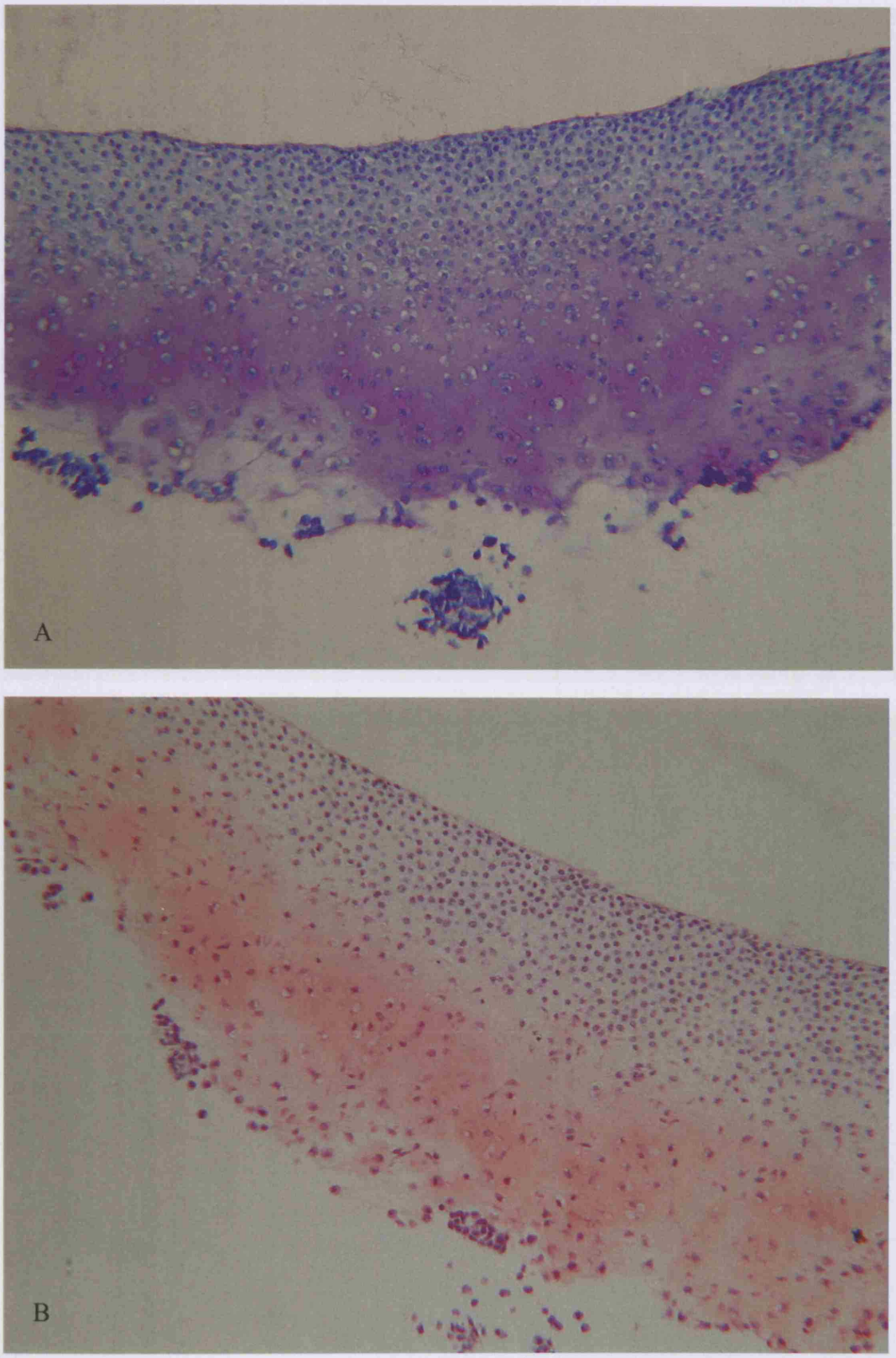



**Figure 3.5** Photomicrographs of pellet cultures prepared using bovine monolayer-expanded chondrocytes. The pellets were cultured for 14 days in DMEM/10%FCS. (A) Toluidine blue staining. (B) Safranin O staining. Magnification x 132.

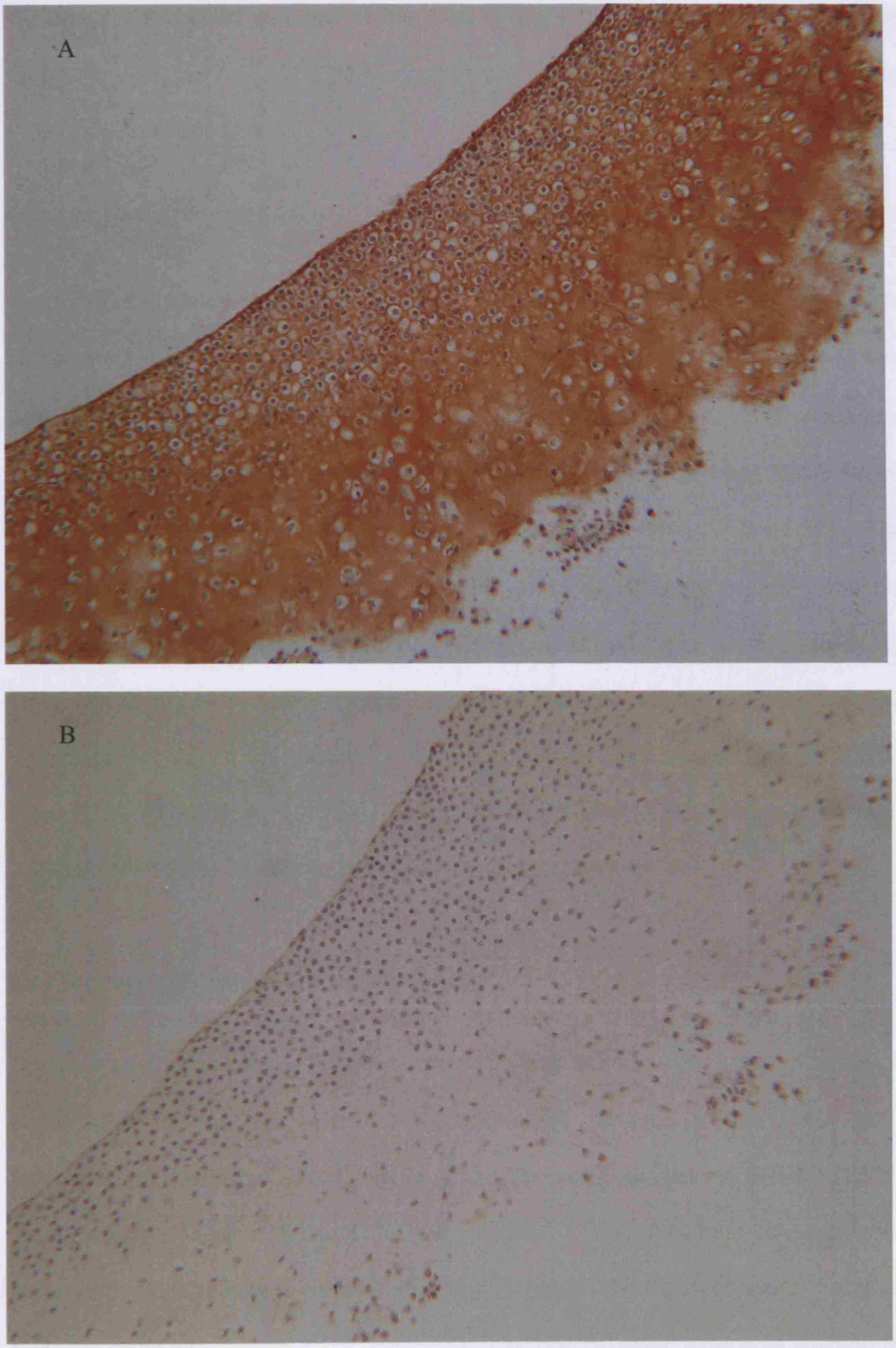



**Figure 3.6** Micrographs representing immunolocalisation for (A) type II and (B) type I collagens from pellet cultures prepared using bovine monolayer-expanded chondrocytes. The pellets were cultured for 14 days in DMEM/10%FCS. Magnification x 132.

By day 7, the pellets cultured in the DMEM/ITS+/TGFβ1 group were noticeably larger than the corresponding pellets cultured in the DMEM/10%FCS group. These differences remained evident at day 14.

**Biochemical Analysis**

Figure 3.9 illustrates total GAG (Figure 3.9A) and DNA (Figure 3.9B) for pellets cultured for two days in DMEM/ITS+/TGF$\beta_1$ and for up to 12 days in either DMEM/ITS+/TGF$\beta_1$ or DMEM/10%FCS. A slight reduction in GAG content was observed during 12 days culture in DMEM/10%FCS. By contrast, GAG levels increased markedly within pellets cultured for 12 days in DMEM/ITS+/TGF$\beta_1$. The mean GAG value of the DMEM/ITS+/TGF$\beta_1$ was significantly greater than the corresponding value for the DMEM/10%FCS group at both days 7 and 14 ($p<0.05$). DNA levels remained fairly static in pellets cultured in DMEM/10%FCS, but increased throughout the culture period for pellets cultured in DMEM/ITS+/TGF$\beta_1$. Accordingly DNA values were significantly greater at day 14 for pellet maintained in DMEM/ITS+/TGF$\beta_1$ compared to DMEM/10%FCS ($p<0.05$).

**Morphology and immunolocalisation**

Gross morphology and standard histology confirmed that cells cultured in DMEM/ITS+/TGF$\beta_1$ generated, macroscopically, larger pellets than those maintained in DMEM/10%FCS (Figure 3.10). The cells cultured in DMEM/10%FCS were associated with reduced ECM, as indicated by toluidine blue staining, when compared to pellets maintained in DMEM/ITS+/TGF$\beta_1$, which stained metachromatically (Figure 3.10). Immunolocalisation revealed extensive staining for type II collagen in pellets cultured in DMEM/ITS+/TGF$\beta_1$ (Figure 3.11B). By contrast, limited type II collagen staining was apparent in the pellets maintained in DMEM/10%FCS (Figure 3.11A). Observations for type I collagen

immunolocalisation revealed reverse trends, with positive staining evident within pellets maintained in DMEM/10%FCS (Figure 3.12A), yet minimal staining observed in the pellets cultured in DMEM/ITS+/TGF$\beta_1$ (Figure 3.12B).

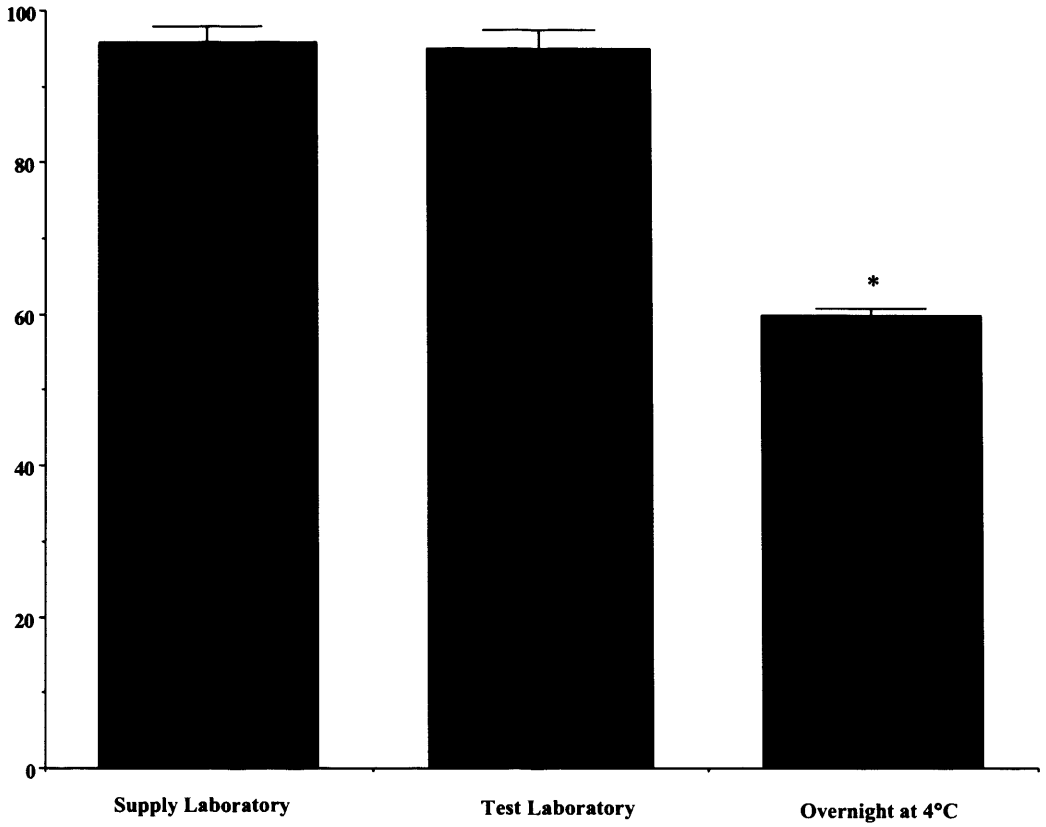



**Figure 3.7** Cell viability (%) of cells from supply laboratory (n=24), test laboratory (n=20) and those incubated overnight at 4°C (n=4)*.

Electron microscopy of the pellets showed obvious cytoarchitectural differences between the two groups. The pellets cultured in DMEM/10%FCS were highly compacted without any obvious spacious matrix (Figure 3.13A). Within the cells clusters of lipid filled vacuoles were apparent and there was limited evidence of collagen fibrils within the pericellular region (Figure 3.13A). By contrast, abundant matrix and collagen fibrils were observed in the pericellular region of pellets cultured in DMEM/ITS+/TGF$\beta_1$ (Figures 3.13B & C).

**Table 3.1** Absolute source and test data from 24 patients undergoing ACI. Included are the cell numbers ($x10^6$) and viability (%).

| | | Supply Laboratory | | | | Test Laboratory | | |
|---|---|---|---|---|---|---|---|---|
| Patient | Age | Cell Count $x10^6$ | Number Vials | Cell Count/vial | Viability % | Cell Count $x10^6$ | Viability | Comments |
| Patient 1 | 49 | 25.90 | 5 | 5.18 | 97.00 | 4.18 | 95.70 | |
| Patient 2 | 36 | 15.00 | 3 | 5.00 | 94.00 | 4.69 | 93.30 | |
| Patient 3 | 24 | 10.50 | 2 | 5.25 | 94.00 | 4.82 | 95.40 | |
| Patient 4 | 35 | 15.00 | 3 | 5.00 | 93.00 | 3.60 | 95.00 | |
| Patient 5 | 33 | 15.00 | 3 | 5.00 | 93.00 | 3.00 | 94.00 | |
| Patient 6 | 40 | 15.00 | 3 | 5.00 | 97.00 | 2.50 | 95.00 | |
| Patient 7 | 31 | 12.30 | 3 | 4.10 | 95.00 | 3.30 | 88.00 | |
| Patient 8 | 40 | 12.00 | 3 | 4.00 | 98.00 | 3.72 | 59.90 | left overnight |
| Patient 9 | 33 | 15.00 | 3 | 5.00 | 98.00 | 4.10 | 60.15 | left overnight |
| Patient 10 | 32 | 27.60 | 6 | 4.60 | 96.00 | 2.30 | 60.00 | left overnight |
| Patient 11 | 23 | 19.20 | 4 | 4.80 | 92.00 | 2.50 | 60.00 | left overnight |
| Patient 12 | 33 | 15.00 | 3 | 5.00 | 98.00 | 4.80 | 97.80 | |
| Patient 13 | 33 | 15.00 | 3 | 5.00 | 96.00 | 3.40 | 95.20 | |
| Patient 14 | 34 | 20.00 | 4 | 5.00 | 97.00 | 3.68 | 96.10 | |
| Patient 15 | 19 | 17.40 | 3 | 5.80 | 99.00 | 3.40 | 99.00 | |
| Patient 16 | 18 | 15.00 | 3 | 5.00 | 97.00 | 3.50 | 93.00 | |
| Patient 17 | 49 | 15.00 | 3 | 5.00 | 98.00 | 4.90 | 96.00 | |
| Patient 18 | 40 | 25.00 | 5 | 5.00 | 94.00 | 3.00 | 98.00 | |
| Patient 19 | 39 | 15.00 | 3 | 5.00 | 97.00 | 5.00 | 98.00 | |
| Patient 20 | 34 | 25.00 | 5 | 5.00 | 96.00 | 4.40 | 97.00 | |
| Patient 21 | 46 | 21.96 | 4 | 5.49 | 91.00 | 4.00 | 98.00 | |
| Patient 22 | 27 | 15.00 | 3 | 5.00 | 96.00 | 3.60 | 95.00 | |
| Patient 23 | 22 | 20.00 | 4 | 5.00 | 98.00 | 3.20 | 95.70 | |
| Patient 24 | 37 | 11.00 | 2 | 5.50 | 95.00 | 4.10 | 96.00 | |

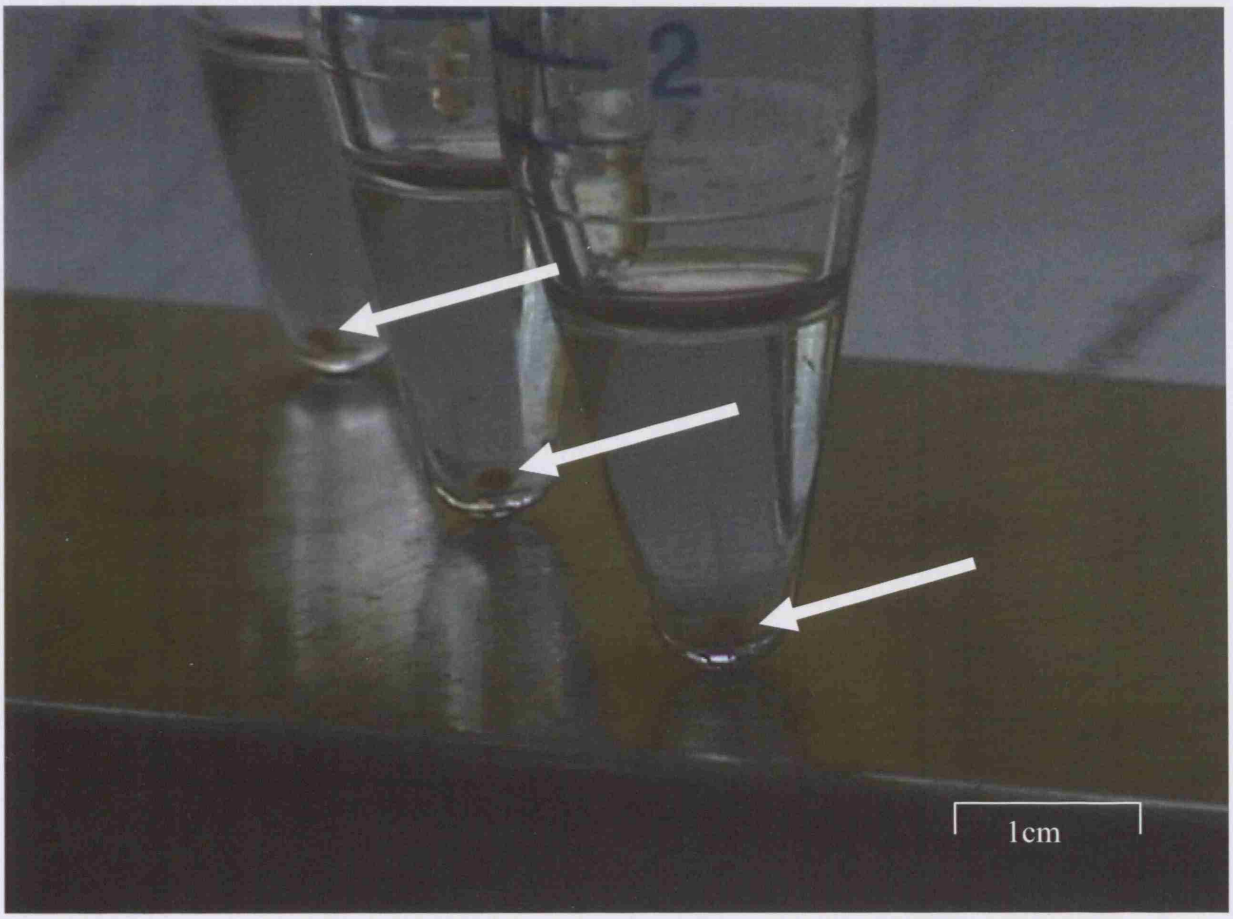



**Figure 3.8** Photograph indicating the gross morphology of chondrocyte pellets (indicated by arrows), prepared with $4x10^5$ cells, and cultured in DMEM/ITS+/TGF$\beta_1$ for 7 days.

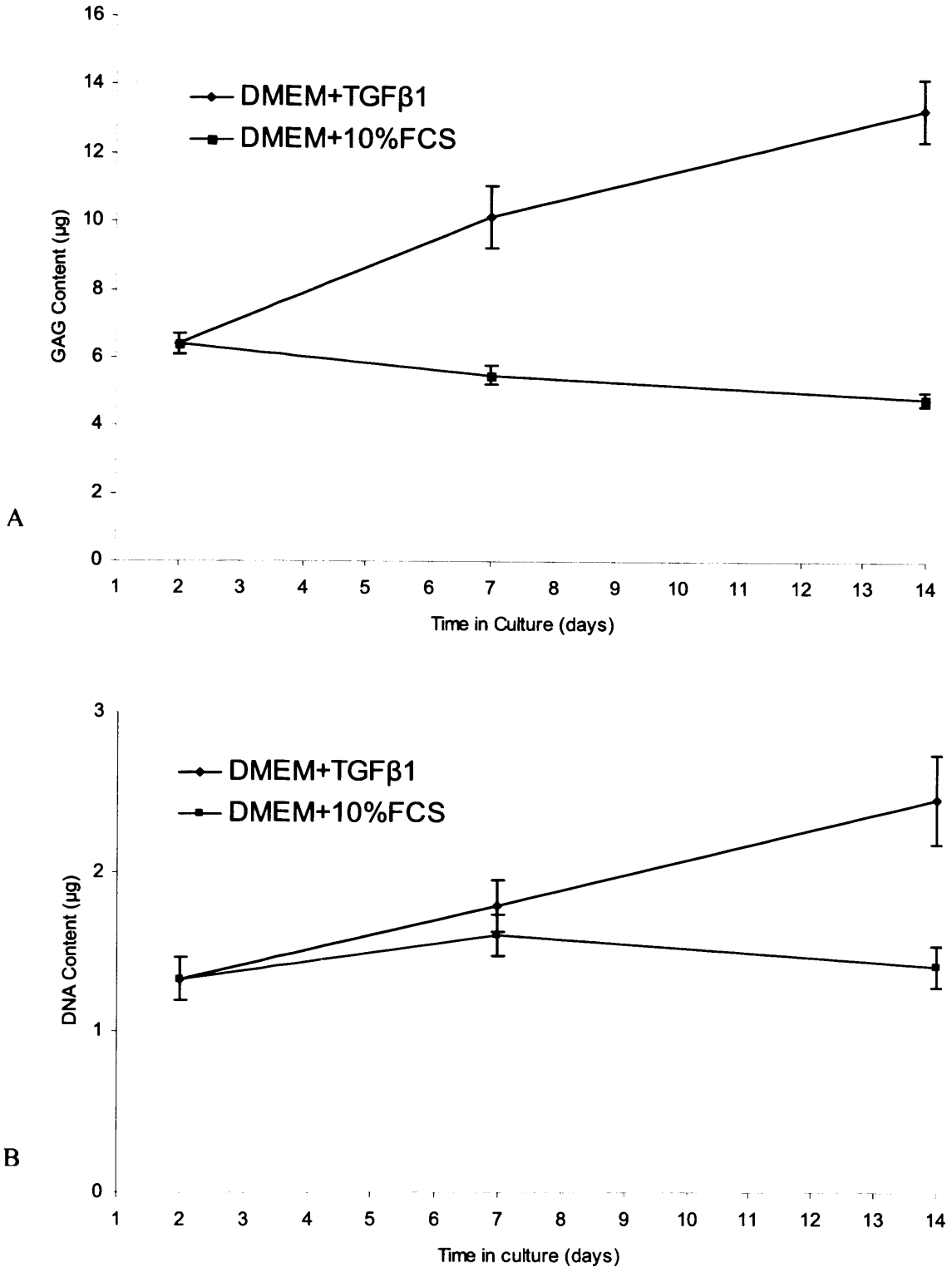



**Figure 3.9** GAG content (A) and DNA content (B) of pellet cultures prepared using human monolayer-expanded chondrocytes. The pellets were cultured for 2 days in DMEM/ITS+/TGF$\beta_1$ and subsequently maintained for up to 12 days in DMEM/ITS+/TGF$\beta_1$ or DMEM/10%FCS. Each point represents the mean and standard error of nine replicates from three separate experiments.

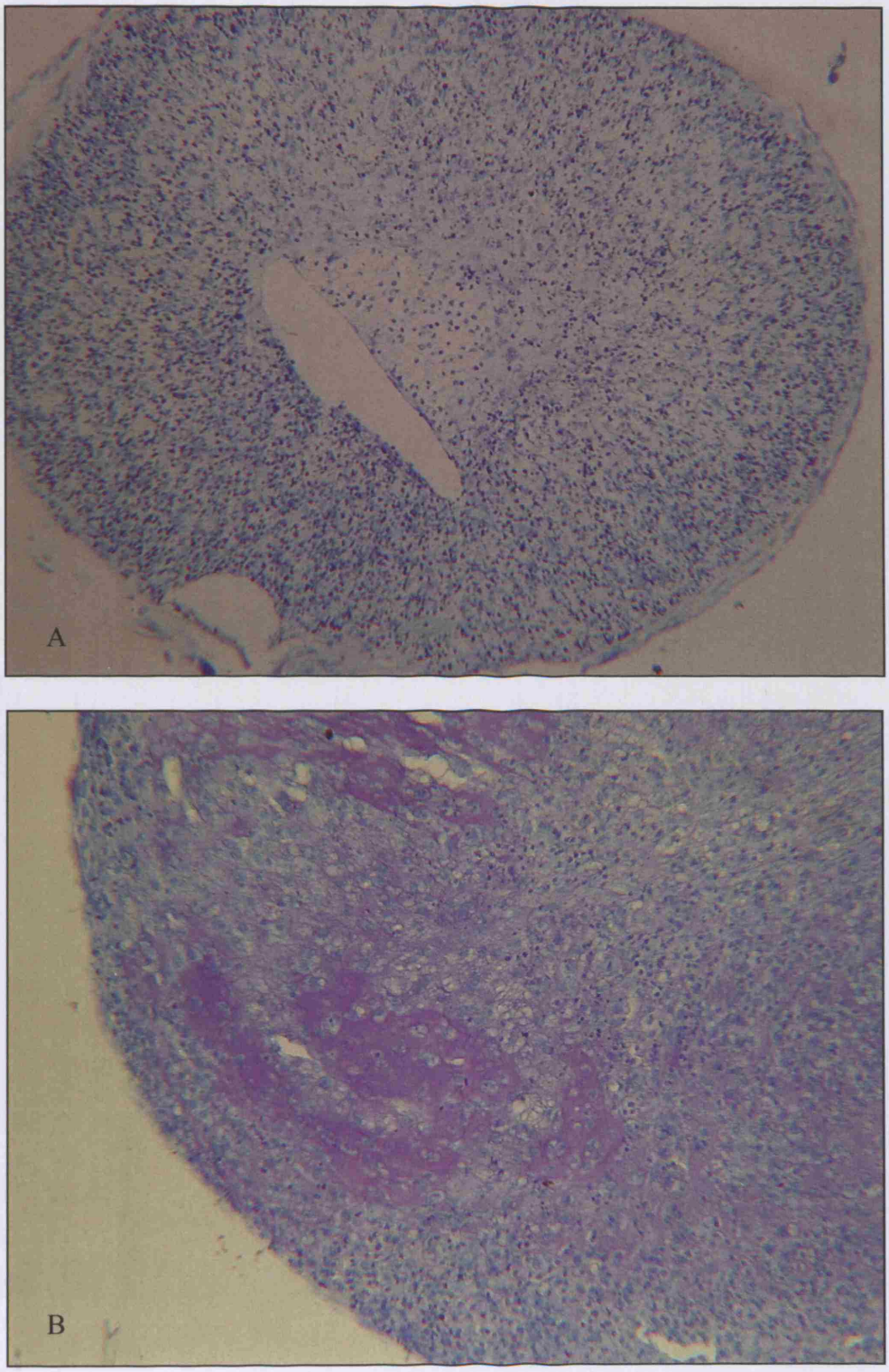



**Figure 3.10** Photomicrographs of pellet cultures prepared using human monolayer-expanded chondrocytes. The pellets were cultured for 2 days in DMEM/ITS+/TGF$\beta_1$ and subsequently maintained for 12 days in DMEM/10%FCS (A) or DMEM/ITS+/TGF$\beta_1$ (B). Toluidine blue staining. Magnification x 132.

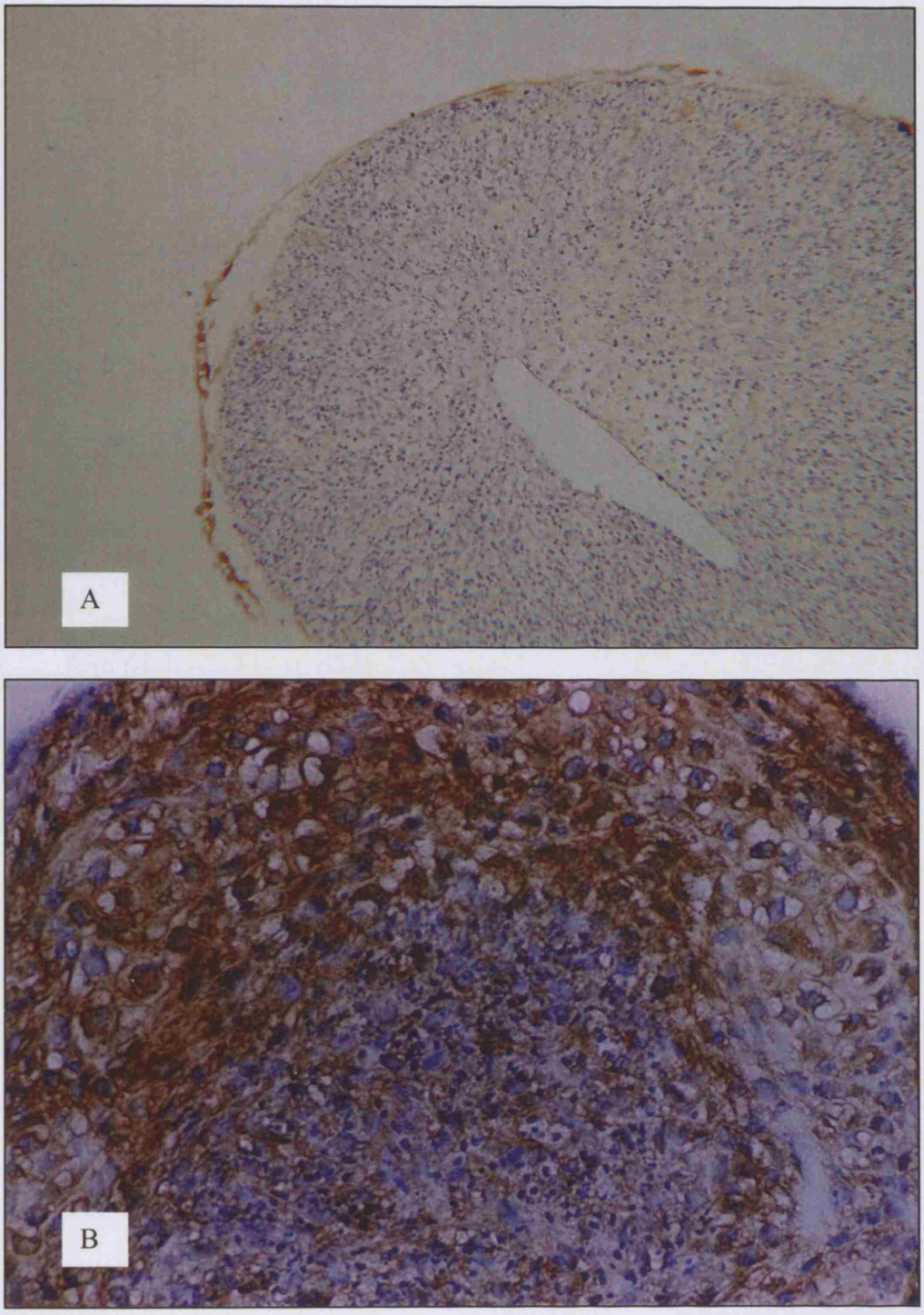



**Figure 3.11** Micrographs representing immunolocalisation for type II collagen from pellet cultures prepared using human monolayer-expanded chondrocytes. The pellets were cultured for 2 days in DMEM/ITS+/TGF$\beta_1$ and subsequently maintained for 12 days in DMEM/10%FCS (A) or DMEM/ITS+/TGF$\beta_1$ (B). Magnification x 132

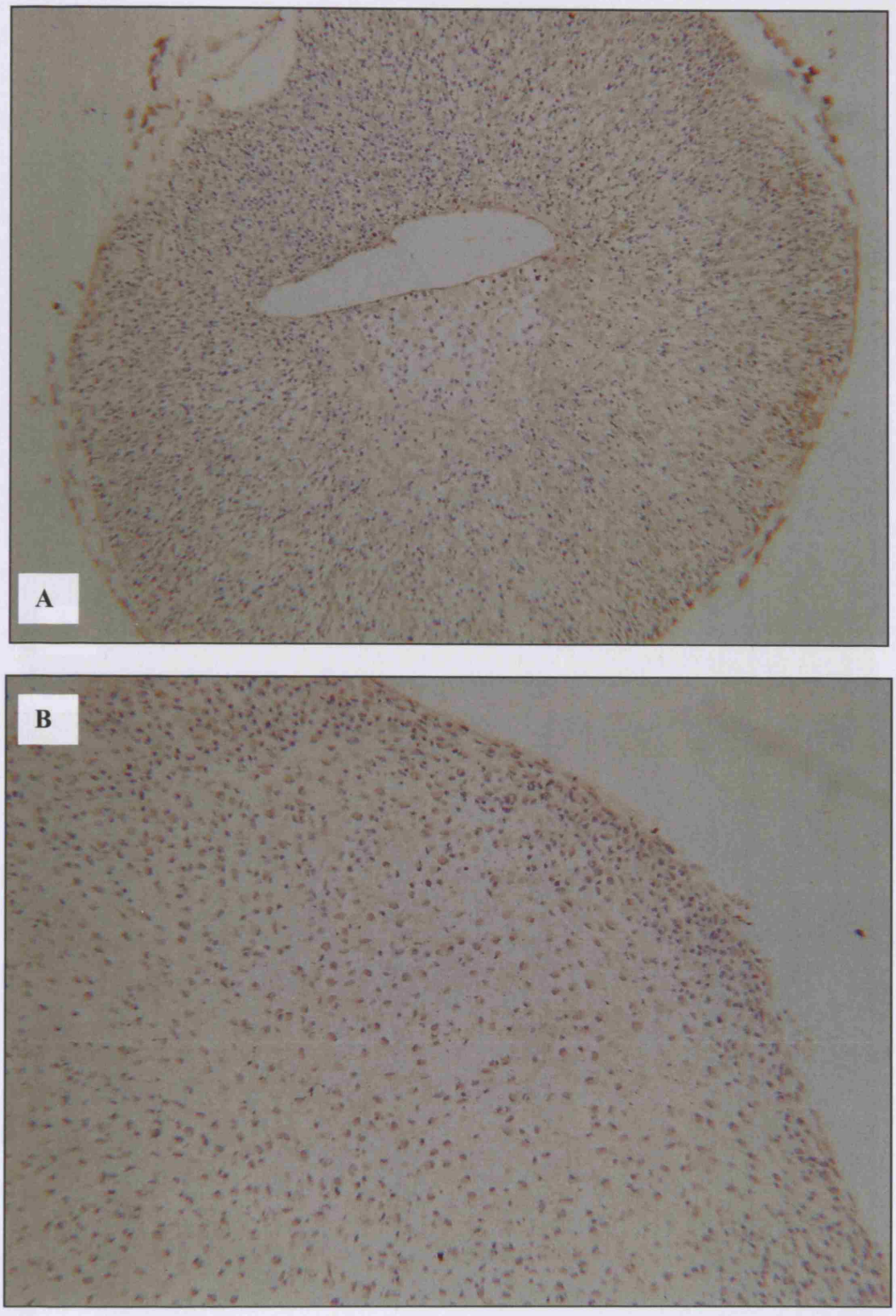



**Figure 3.12** Micrographs representing immunolocalisation for type I collagen from pellet cultures prepared using human monolayer-expanded chondrocytes. The pellets were cultured for 2 days in DMEM/ITS+/TGF$\beta_1$ and subsequently maintained for 12 days in DMEM/10%FCS (A) or DMEM/ITS+/TGF$\beta_1$ (B). Magnification x 132.

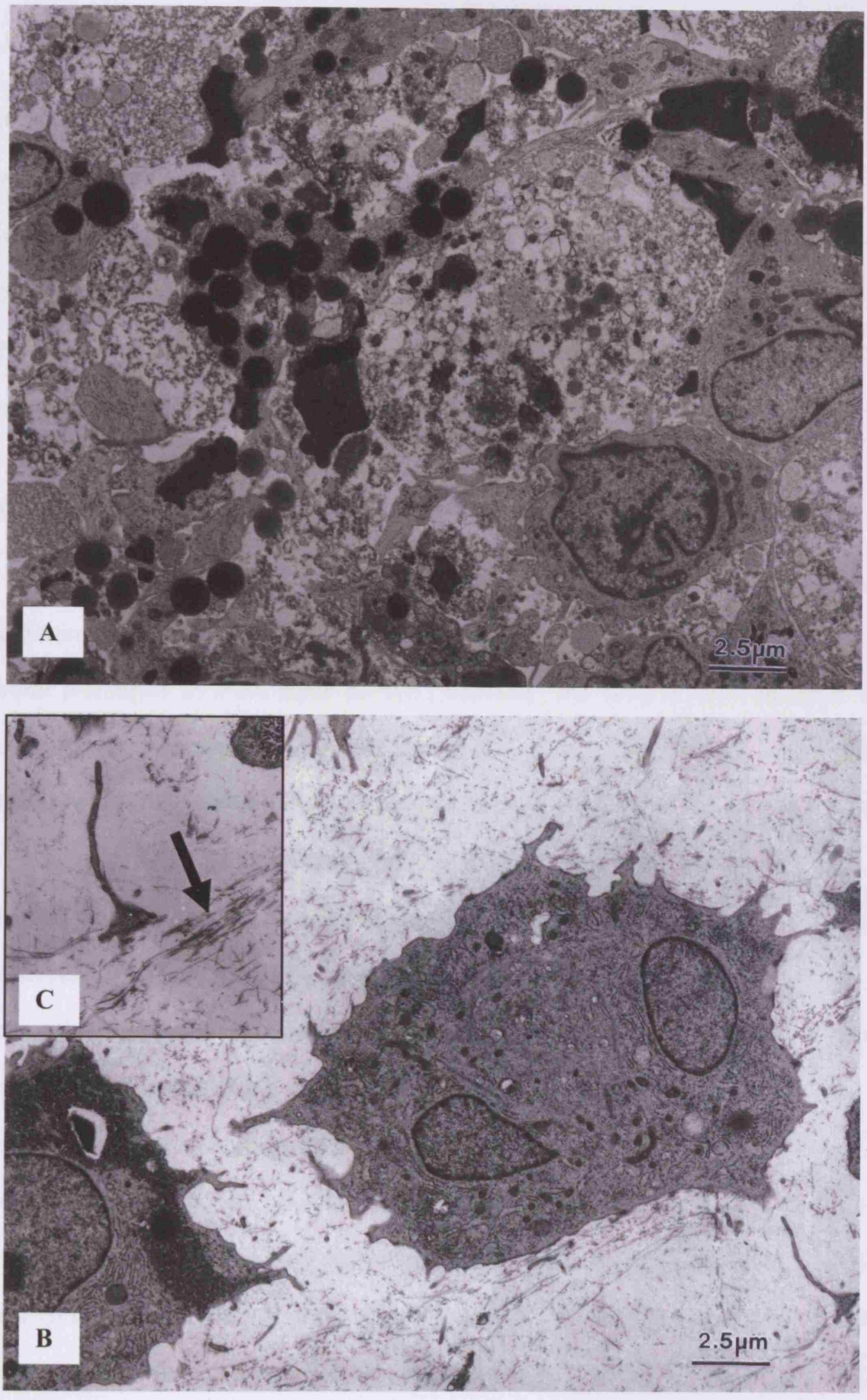



**Figure 3.13** Electron micrographs of pellet cultures prepared using human monolayer-expanded chondrocytes. The pellets were cultured for 2 days in DMEM/ITS+/TGF$\beta_1$ and subsequently maintained for 12 days in (A) DMEM/10%FCS (x6000) or (B) DMEM/ITS+/TGF$\beta_1$ (x6000). Sub-figure (C) is taken from the bottom right hand corner of Figure (B) (x26000). This indicates the presence of collagen fibrils in the ECM (arrow).

## 3.4 Discussion

The studies described in this chapter were designed to assess the ability of monolayer expanded chondrocytes to redifferentiate when transferred into a 3D environment. Accordingly it tested the hypothesis that a TGFβ1-containing defined culture medium, enhances the re-expression of a chondrocytic phenotype and the subsequent production of cartilaginous ECM by human chondrocytes used in the clinical practice of ACI.

**Bovine Chondrocytes**

In the current study it was found that bovine chondrocytes appeared dedifferentiated under light microscopy within one passage in monolayer. After several passages in monolayer culture, however, the cells readily reverted to a chondrogenic phenotype when transferred into a 3D environment. In pellet culture in DMEM+10%FCS, DNA values increased by 67% between days 2 to 14 ($p>0.05$) and GAG synthesis was stimulated within pellets cultured in DMEM/10%FCS, as indicated by a 7% increase in GAG levels between days 2 and 14, which was statistically significant ($p<0.05$). It must be noted that total GAG was not measured and we only measured GAG in the pellet and not in the medium and these results might be confounded if the GAG was being produced but then released into the medium. Another interpretation is that that the cells began synthesising proteoglycans as soon as they are pelleted, and by day 2, the amount of GAG was already quite high, increasing only moderately thereafter. Histological data confirm these findings and reveal a remarkable appearance almost resembling the gross morphology of articular cartilage with the presence of a superficial, middle and deep zone. The concentrated cells on one side of the composite resembled the superficial zone, whereas the sparse cells with large amounts of glycosaminoglycan as indicated by extensive metachromatic staining (Figure 3.5A) on the opposing

side resembled the deep zone. Interestingly, the pellet attempts to form a spherical shape and this may have resulted in the cells on one side of the tissue to be compressed, whereas the cells on the outer surface may be subjected to tension forces.

Freshly extracted bovine chondrocytes, have previously been reported to display the ability to form a zonal structure, when placed in micromass culture (Hall *et al.*, 2004). However, in contrast, cells that had undergone successive passages, revealed disorganised grafts. The current findings differ in that cells that were passaged three times prior to pelleting, revealed an organised structure. Two possible explanations exist. One may involve the self selection of articular cartilage progenitor cells in different parts of the pellet (Bishop, 2004). Alternatively the cell population may be homogeneous in nature and merely respond to compression-tension forces in the floating pellet.

**Human Autologous Chondrocytes**

To the author's knowledge, the present work represents the first independent audit of the numbers and viability of the cells used in the clinical practice of ACI. The procedure for the provision of expanded chondrocytes for implantation surgery involves the transportation of the cells in a proprietary transport medium using an overnight courier. The present data indicate that cell numbers were slightly reduced compared to those documented at the supply laboratory (Table 3.1). The differences are probably due to cell sedimentation and entrapment around the vial cap, leading to a slightly reduced count. Nonetheless the cell viability was maintained at approximately 95% following transportation (Figure 3.7). By contrast, when cells were stored in their medium at 4°C overnight, cell viability was reduced significantly. This finding supports the recommendations of the service provider that cells must be implanted as soon as possible after delivery.

Cells were expanded in monolayer culture using autologous serum. Results indicated a consistent pellet formation and redifferentiation of the cells cultured in DMEM/ITS+/TGF$\beta_1$ medium. In preliminary experiments, it was found that the human passaged chondrocytes that had been monolayer cultured in autologous serum failed to form pellets when centrifuged and cultured in DMEM/10%FCS. This finding occurred despite the presence of serum-derived adhesion factors within the medium, indicating the importance of the growth factor environment on the ability of cells to form pellets. This is the reason why, subsequently, all cells were exposed to the DMEM/ITS+/TGF$\beta_1$ medium in the first instance, to allow pellets to form. However, it was also found that if these passaged cells were then monolayer cultured in DMEM+10%FCS for one further passage then the cells would subsequently pellet in the DMEM/10%FCS medium. This finding supports the recent proposition that, the conditions of expansion of human articular chondrocytes can modulate their ability to re-enter the differentiation programme upon transfer into a 3D environment (Jakob *et al.*, 2001). Accordingly, the expression of specific adhesion molecules or surface markers may be altered by the culture conditions used during monolayer expansion as suggested previously (Chimal-Monroy and Diaz de Leon, 1999). However, it is important to re-iterate that histological analysis of these pellets did not reveal any evidence of chondrogenesis and therefore although the cells were capable of forming pellets, they were not able to redifferentiate in the absence of other factors.

Jacobs *et al* assessed the effects of TGFβ on human chondrocytes taken from the ankle or hip and expanded in serum supplemented with fetal calf serum (Jakob *et al.*, 2001). They investigated monolayer and pellet culture and questioned the previous theory that the effects of TGFβ were solely due to its ability to stimulate cells which possessed a rounded morphology (Trippel, 1995; Yaeger *et al.*, 1997). More recent reports have suggested a synergistic action between TGFβ and insulin

or IGF-I stimulating the expression of chondrocytic markers by both human articular chondrocytes (Yaeger *et al.*, 1997) and rabbit auricular chondrocytes (van Osch *et al.*, 2001).

The results presented in the current study are in agreement with these studies, and confirm that the use of a defined medium which contains both TGFβ1 and insulin enhances the re-expression of a chondrocytic phenotype within pellet culture. Thus pellets maintained in DMEM/10%FCS demonstrated a limited re-expression of the chondrocytic marker, collagen type II, and continued to produce type I collagen throughout the culture period (Figures 3.11A and 3.12A). By contrast, pellets cultured in DMEM/ITS+/TGFβ$_1$ demonstrated significant labelling for collagen type II (Figure 3.11B), while collagen type I expression was minimal (Figure 3.12B). As noted in Figure 3.11, the cells toward the outside of the pellet displayed a hypertrophic appearance, with larger nuclei, which is why there appears to be a stark difference in cell size between those cells cultured in DMEM/10%FCS (Figure 3.11A) and those cultured in DMEM/ITS+/TGFβ$_1$ (Figure 3.11B).

The repair of chondral defects using ACI techniques requires the proliferation of cells and the elaboration of a GAG-rich ECM. Thus it is important to note, in addition to the alteration in the expression of phenotypic markers, that pellets cultured in DMEM/ITS+/TGFβ$_1$ were larger with greater amounts of DNA and GAG compared to pellets maintained in DMEM/10%FCS. DNA values increased by 80% from days 2 to 14, suggesting that DMEM/ITS+/TGFβ$_1$ has a mitogenic effect on the monolayer expanded human cells. This level of proliferation is broadly similar to that reported previously for primary human chondrocytes cultured in alginate beads over a 4 week period (Almquvist *et al.*, 2001). By contrast, DNA values did not increase significantly between days 2 and 14 for pellets maintained in DMEM/10%FCS, indicating a limited mitogenic potential provided by this medium (Figure 3.9B).

GAG synthesis was stimulated within pellets cultured in DMEM/ITS+/TGF$\beta_1$, as indicated by a 107% increase in GAG levels between days 2 and 14. By contrast GAG levels were reduced by 25% over the same period for pellets maintained in DMEM/10%FCS. As noted previously, GAG in the medium was not measured and so this data only represents the GAG measured in the pellets themselves and not the total GAG in the system. Histological and electron microscopic data, however, confirm the suggestion that pellets maintained in DMEM/ITS+/TGF$\beta_1$ possessed an extensive ECM (Figure 3.10B), containing clearly defined collagen fibrils (Figures 3.13B and 3.13C). Conversely, pellets maintained in DMEM/10%FCS possessed limited ECM (Figure 3.10A) and the cells were small, approximately 10 μm in diameter, and laden with lipid filled vacuoles (Figure 3.13A), suggesting that they were less active than those cultured in DMEM/ITS+/TGF$\beta_1$.

Previous studies, reporting the effects of TGFβ on GAG synthesis by chondrocytes, are somewhat ambiguous (Glansbeek *et al.*, 1998; van Osch *et al.*, 1998; Yaeger *et al.*, 1997; van Beuningen *et al.*, 1994; Benya and Padilla, 1993; van der Kraan *et al.*, 1992b; Skantze *et al.*, 1985). For example, one of these studies describes an inhibition of proteoglycan synthesis in the presence of TGFβ in freshly isolated chondrocytes, but a stimulation in cells that have undergone monolayer expansion (van der Kraan *et al.*, 1992b). Accordingly, the authors suggested that the effect was dependent on the stage of chondrocytic differentiation. By contrast, another study reported a stimulatory effect that was independent of the stage of differentiation of the cells (van Osch *et al.*, 1998). One recent study proposed that the establishment of a pericellular matrix and intercellular separation may be a requisite for stable expression of the articular chondrocyte phenotype (Stewart *et al.*, 2000), in agreement with the "chondron" model (Poole *et al.*, 1987).

Moreover, Jakob and colleagues showed enhanced dedifferentiation of chondrocytes when exposed to FGF-2/TGFβ in monolayer, yet enhanced re-differentiation and production of cartilaginous matrix in pellet culture on exposure to TGFβ (Jakob *et al.,* 2001). These differences can be attributed to the variation of experimental protocols between studies, involving differences in cell type, model system and the concentration of growth factors (van Beuningen *et al.*, 1994; van der Kraan *et al.*, 1992a; van Osch *et al.*, 1998). However all of these studies support the current findings related to the effect of TGFβ on GAG synthesis by human monolayer expanded chondrocytes.

In the present study, cells were expanded in monolayer using culture medium supplemented with autologous serum. The original description of the ACI technique on 23 patients also used autologous serum during monolayer culture (Brittberg *et al.*, 1994b). This practice however, has not subsequently been standardised in clinical use, where supplementation with either autologous serum or fetal calf serum are employed by different companies supplying the cells, and this could have important clinical consequences. One of the reasons that certain commercial manufacturers justified using media supplemented with bovine serum was that it contains FGF, which is though to be a potent mitogen. The results of the present study demonstrated that cells cultured in fetal calf serum failed to aggregate and re-differentiate which therefore, does not support this argument.

The present study confirms that a defined medium containing TGFβ1 induces the re-expression of a chondrocytic phenotype and the subsequent stimulation of GAG and type II collagen production by human monolayer expanded chondrocytes. Accordingly, TGFβ may be necessary for the formation of cellular aggregates, the initiation of cell proliferation and the development of a hyaline cartilage matrix within a clinical repair situation.

# CHAPTER FOUR – ALTERNATIVE CELL SOURCES

## 4.1 Introduction

In the procedure of ACI it is common practice to harvest the donor cells from non-articulating or non load-bearing areas of the knee joint, such as the medial border of the medial femoral condyle, the intercondylar notch and occasionally the lateral border of the lateral femoral condyle. One limitation of this strategy is that normal tissue is sacrificed in order to perform a repair procedure, potentially causing further damage to the affected knee.

During ACI, the cored out defect (damaged tissue) is normally discarded. However, histology of this defect revealed areas of hyaline cartilage interspersed with fibrous tissue. It is, therefore, feasible that the cored out tissue could contain sufficient chondrocytes to be used for the purposes of ACI.

Bone marrow contains a multipotential stromal cell known as colony-forming unit-fibroblasts (CFU-F), which are capable of differentiating into chondrocyte (Johnstone *et al.,* 1998; Yoo *et al.*, 1998), osteoblasts (Goshima *et al.*, 1991; Lennon *et al.*, 1995; Beresford *et al.*, 1994), fibroblasts (Golde *et al.*, 1980) and adipocytes (Beresford *et al.,* 1992). Differentiation is controlled by various environmental cues, including growth factors and related morphogens and mechanical factors.

It has long been recognised that mobilization and differentiation of these bone-marrow-derived mesenchymal progenitor cells have played a role in the repair of damaged articular cartilage (Insall, 1967). In addition, the importance of bone marrow components was reinforced by the lack of repair of partial thickness defects

of articular cartilage, where the subchondral bone is not penetrated (Hunziker *et al.,* 1996; Buckwalter, 1995; Fuller and Ghadially, 1972).

Wakitani displayed differentiation of mesenchymal progenitor cells within cartilage defects *in vivo* (Wakitani *et al.,* 1994b). *In vitro*, a system has been described that allows direct study of the differentiation process from stem cells to chondrocytes both in rabbits (Johnstone *et al.*, 1998) and humans (Yoo *et al.*, 1998). These models utilise the "pellet" culture system originally described as a method for preventing the phenotypic modulation of chondrocytes *in vitro* (Holtzer *et al.*, 1960; Manning, Bonner, 1967) and allows assessment of the factors that regulate the progression of cells through the chondrogenic lineage.

In this chapter two possible alternative sources of cells for reparative procedures are examined. In one study the possibility of harvesting cells from the defect is assessed and in the second study the author aims to replicate the above studies to examine whether mesenchymal progenitor cells harvested from young human subjects could be isolated and differentiated into chondrocytes capable of being used in clinical practice.

## 4.2 Materials & Methods

In this chapter two alternate sources of cells are investigated, human defect derived chondrocytes (HDDC) and mesenchymal progenitor cells.

### 4.2.1 Isolation of HDDC

During the second stage operative procedure of ACI, the surgeon dissects out the cartilage defect down to, but not through, the subchondral bone plate (so not to induce bleeding) leaving stable healthy edges of cartilage (Figure 2.3). In

preliminary experiments, pieces of damaged cartilage from 10 patients were placed into formalin and analysed using histology. The defect, sized 10x20mm, from one male patient aged 36 years, was transferred in a sterile container containing normal saline to the culture laboratory for processing. The cartilage was diced into smaller pieces (approximately 2mm x 2mm) to improve enzymatic digestion and incubated at 37°C for 1 hour in 10mL of DMEM + 10%FCS + 700 unit.mL$^{-1}$ pronase (BDH Ltd, Poole, UK). The supernatant was removed and replaced with 20mL of DMEM+10%FCS+100unit.mL$^{-1}$ collagenase type Ia (Sigma, Poole, UK) and incubated for 16 hours overnight at 37°C on a rolamixer.

The next day the supernatant containing the released chondrocytes was passed through a 70 μm pore size cell sieve (Marathon Laboratory Supplies, London, UK) and the filtrate was centrifuged at 2000 x g for 7 minutes. The supernatant was aspirated and the cells were washed by two further resuspensions in 10mL DMEM+10%FCS. A cell count and viability was performed using the trypan blue dye exclusion test (Fong and Kissmeyer-Nielsen, 1972) and the cells were plated in tissue culture flasks at an initial seeding density of 1 x $10^4$ cells/cm$^2$ (175 cm$^2$ growth area, Falcon, Oxford, UK) in 35mL of DMEM+10%FCS. The cells were maintained at 37°C/5% $CO_2$ in DMEM + 10%FCS and the culture media was changed every two days. After 7 days in culture, the chondrocytes were rinsed twice in phosphate buffered saline pH 7.4 (PBS, Sigma, Poole, UK) and recovered by incubation for 15 min in 0.25% (w/v) trypsin (Sigma, Poole, UK) in PBS. The cells were re-seeded into 175cm$^2$ flasks containing 35mL DMEM/10%FCS at 37°C/5%$CO_2$ for further expansion and taken through 3 further passages, as described previously.

At each passage, the number of cells recovered and cell viability was determined using a haemocytometer and the trypan blue exclusion test. Expansion of cells was calculated using Equation 1 below (Wiseman *et al.*, 2004).

$$\text{Cell expansion}_{(p_n - p_{n+1})} = \frac{\text{Viable cell number recovered}_{(p_{n+1})}}{\text{Cell number seeded}_{(p_n)}} \quad \text{.....Equation 1.}$$

$p_n$ = passage number

**Pelleting of HDDC**

After 3 passages the cells were counted and viability was assessed using the trypan blue dye exclusion test (Fong and Kissmeyer-Nielsen, 1972). Half the cells were used for pelleting experiments and half the cells were replated for one further passage. For the pelleting experiments, the cells were divided into two groups cultured in either DMEM/10%FCS or in a defined medium, DMEM/ITS+/TGFβ1, as described previously. The medium was changed every 2 days and pellet cultures were removed at days 2, 7 and 14 for subsequent analysis.

### 4.2.2 Harvesting Bone Marrow Samples

Ethics committee approval was obtained (appendix C). Patients being admitted for arthroscopies or second stage cartilage procedures, at the Royal National Orthopaedic Hospital NHS Trust, were approached to donate a small sample of their bone marrow, at the same anaesthetic sitting. Informed consent was obtained. Samples of between 5-12mL bone marrow aspirate were taken from 13 young healthy volunteers (aged 30-41 mean 29 years, 8 male and 5 female). Following anaesthesia, each patient was moved into a right lateral decubitus position. Under aseptic conditions, 5mL of 0.25% bupivicaine local anaesthetic was infiltrated under the skin and down to the periosteum overlying the bony prominence of the posterior

superior iliac spine. A stab incision was then made with a pointed blade and a bone marrow trephine needle was passed through the outer cortical table of the iliac crest into the marrow space. Several heparinised 5mL syringes were connected to the trephine needle and used to aspirate bone marrow contents (each syringe contained 1000U heparin). When the blood stopped flowing freely, the procedure was stopped. An elastoplast dressing was applied over the site and the patient turned back to the supine position.

The sample was transferred into a small flask using a sterile technique, and immediately transported to the laboratory. There have been two reported methods in the literature for isolating mesenchymal progenitor cells from the aspirate. In this chapter both methods were assessed.

**Method 1 – Isolation of stem cells from blood by monolayer culture**

The sample of bone marrow aspirate, harvested as above, was passed through a 19 and 21 gauge syringe and diluted with an equal volume of DMEM/10%FCS. A small 50 μL aliquot was added to 50 μL acetic acid (4%), to disrupt the red blood cells (Wakitani *et al.*, 1994b) and 100 μL trypan blue, for counting on a haemocytometer, on a one to four concentration as described previously. The cells were then plated at a density of approximately $10x10^4$ cells/$cm^2$ in a $75cm^2$ flask, in 10mL DMEM/10%FCS, and incubated at 37°C/5%$CO_2$.

The media was first changed five days after seeding (Wakitani *et al.*, 1994b), and following that the media was changed every four days. The cells were trypsinised and passaged when they reached 80% confluence. Cells were re-plated at a density of $10x10^4$ cells/$cm^2$. The flasks were inspected daily under the microscope. On reaching 80% confluence the cells were trypsinised, counted and either re-plated or pelleted.

**Method 2 – Isolation of Mesenchymal Cells using the Ficoll®-Paque Technique**

The Ficoll® Technique (Amersham Pharmacia Biotech), was developed to allow rapid isolation of lymphocytes from whole blood. The technique works on the principle that Ficoll® is a low viscosity erythrocyte aggregating agent, which is used in a centrifugation procedure to isolate cell types.

The bone marrow sample was passed through 19 and 21 gauge needles and added to an equal mixture of EBSS. 3mL Ficoll® was added to six centrifuge tubes. 4mL mixture incorporating 2mL bone marrow and 2mL EBSS was carefully placed on top of the Ficoll® in the centrifuge tube to ensure the two layers remained separate. The tubes were centrifuged at 400 x g for 30 minutes at 18°C. The plasma forms a top layer and the isolated lymphocytes, form a separate layer below it. The lymphocyte layer was carefully aspirated and placed into a clean centrifuge tube (Falcon, Beckton Dickinson, NJ). The cells were washed by the addition of 10mL EBSS and the mixture centrifuged at 60g for 10 minutes at 18°C. The final cell solution in each of the tubes were finally resuspended together in 10mL DMEM/10%FCS and plated in a 75 $cm^2$ flask.

The media was first changed every four days. The cells were trypsinised and passaged when they reached 80% confluence. Cells were re-plated at a density of $10x10^4 cells/cm^2$. The flasks were inspected daily under the microscope. On reaching 80% confluence the cells were trypsinised, counted and either re-plated or pelleted.

**Method for pelleting the human mesenchymal progenitor cells**

At either passage 2 or passage 3, cells aliquots of $4x10^5$ cells were centrifuged at 500g in 15mL polypropylene conical tubes (Johnstone *et al.*, 1998). The supernatant was removed and the resultant pellet cultures were cultured at

37°C/5%$CO_2$ in a defined medium comprising of Dulbecco's Modified Eagles Medium, ITS+ Premix [Collaborative Biomedical Products: insulin (6.25μg.mL$^{-1}$), transferrin (6.25μg.mL$^{-1}$), selenous acid (6.25μg.mL$^{-1}$), bovine serum albumin (1.25mg.mL$^{-1}$), linoleic acid (5.35μg.mL$^{-1}$)]. Pyruvate (1mM), ascorbate 2-phosphate (37.5 μg.mL$^{-1}$), dexamethasone ($10^{-7}$ M) and 10ng.mL$^{-1}$ TGF$\beta_1$ (recombinant human, R&D Systems) were also added. This medium is designated DMEM/ITS+/Asc/TGF$\beta_1$ hereafter. The medium was changed every 2 days and pellet cultures were removed at various time points up to 14 days for subsequent biochemical (GAG and DNA) and histomorphological analysis (Toluidine Blue, Safranin O and Collagens Types I,II and X), as described previously.

## 4.3 Results

### 4.3.1 Histology of Cartilage Defects

In the biopsies harvested from the defects of young adult males, the surface was typically irregular and discontinuous with evidence of a combination of a fibrocartilaginous cap and some matrix below it resembling hyaline cartilage (Figure 4.1A). There is generally the presence of clefts, which sometimes extends down to the radial (deep) zone (Figure 4.1B/C/D). The cell distribution was often abnormal in the deep zone without the classical columnar appearances and there was evidence of disorganized clusters of cells as can be found in degenerative tissue (Figure 4.1B). Because the tissue had been cored off from the underlying subchondral bone, with a deliberate attempt not to disrupt the subchondral bone, it was, therefore, not possible to assess the integration between the cartilage and the underlying bone.

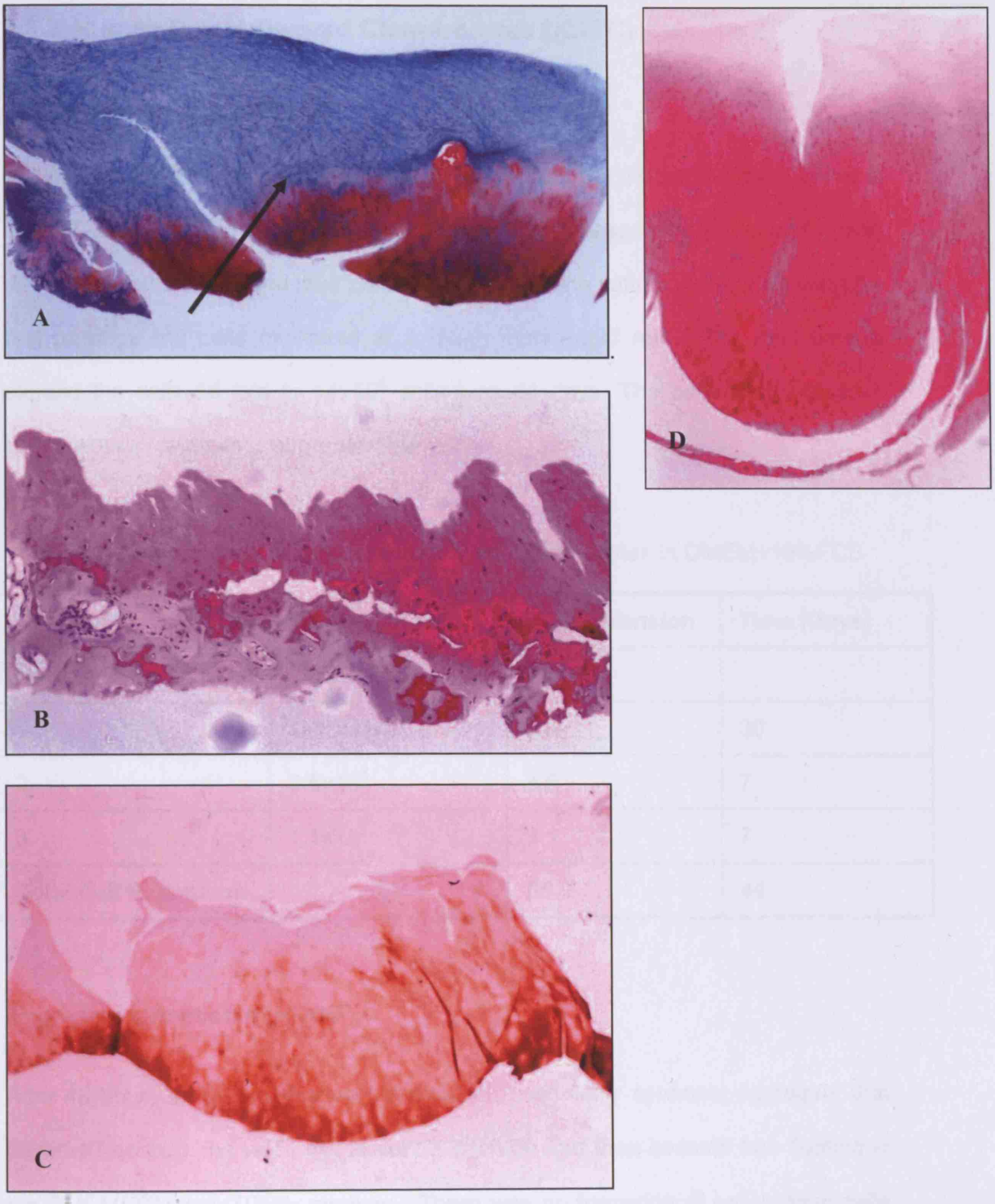



**Figure 4.1** Representative photomicrographs of cored out defect tissues from young patients during the second operative stage of ACI. (A) Masson Trichrome (B) toluidine blue (C) immunolocalisation for type II collagen and (D) safranin O. Magnification x 32. Note the fibrous cap overlying somewhat normal elements of articular cartilage in Figure A (arrow)

### 4.3.2 Human Defect Derived Chondrocytes (HDDC)

#### Cell expansion of HDDC

Table 4.1 indicates the number of cells obtained from the defect and the number of cells at each passage. The length of time to first passage was prolonged mainly due to slow adherence and multiplication of cells on the culture plate. Following the first passage the cells multiplied at a much more rapid rate. The total time to expand the cells 55 fold to $11x10^6$ cells was 44 days. The cell viability at each passage was maintained at greater than 90%.

**Table 4.1** - Expansion of human defect-derived chondrocytes in DMEM+10%FCS

| **Passage** | **Number of cells** | **Cell Expansion** | **Time (Days)** |
|---|---|---|---|
| Harvest (0) | $2x10^5$ | | |
| 1 | $1x10^6$ | 5.0 | 30 |
| 2 | $3.5x10^6$ | 3.5 | 7 |
| 3 | $1.1x10^7$ | 3.1 | 7 |
| **Total Cell Expansion** | | 55.0 | 44 |

#### Aggregate formation of HDDC

After 48 hours in culture, the cells formed an essentially spherical aggregate that became free from the walls of the centrifuge tube and then became free floating in the DMEM/ITS+/Asc/TGF$\beta_1$ medium. There was no formation of pellets from cells cultured in DMEM/10%FCS and after 5 days these samples were discarded.

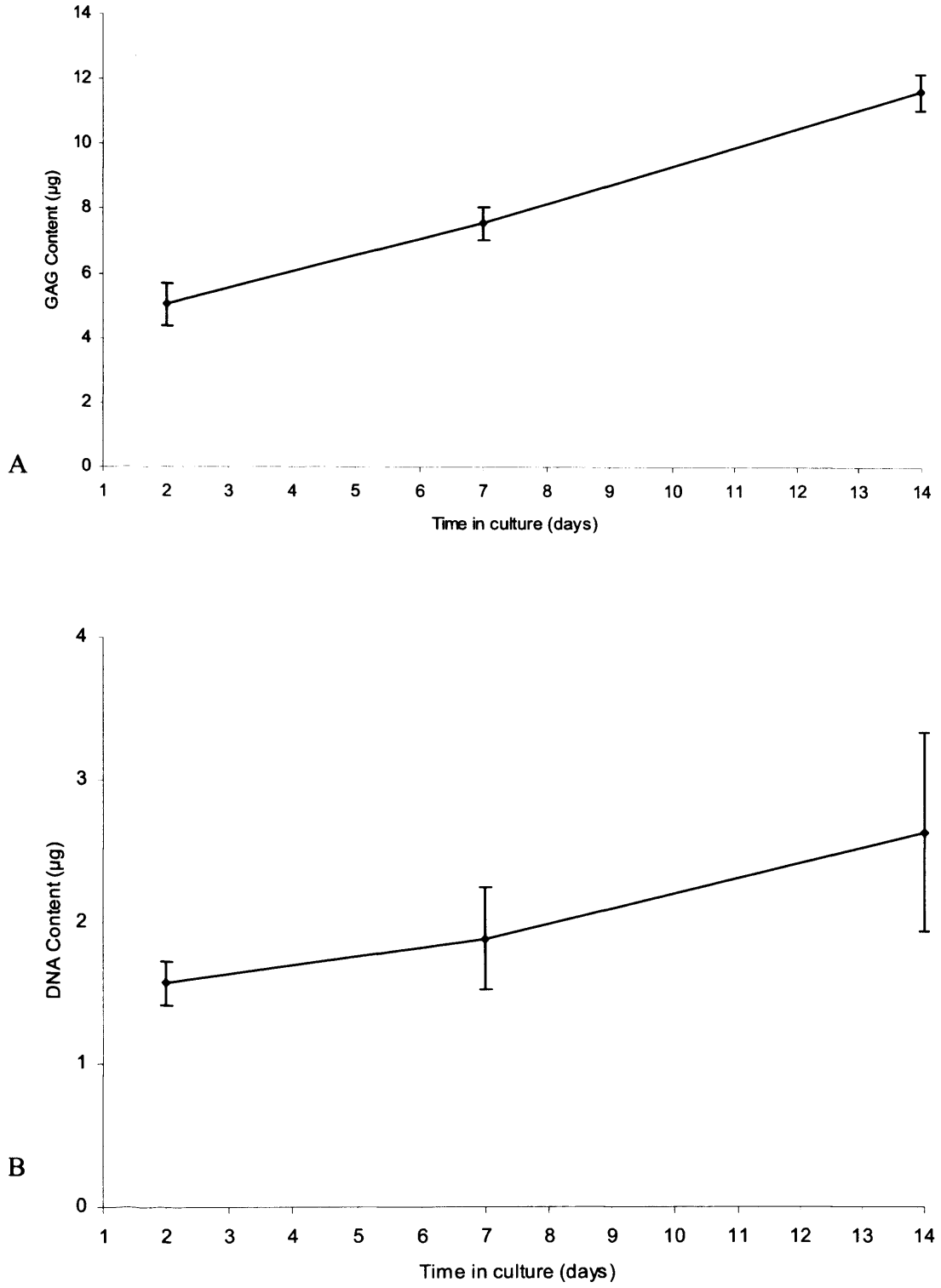



**Figure 4.2.** GAG content (A) and DNA content (B) (±SE) of pellet cultures prepared using human monolayer-expanded chondrocytes derived from the patients chondral defect. The pellets were cultured for 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. Each point represents the mean and standard error of nine replicates.

**Biochemical Analysis**

Figure 4.2 illustrates total GAG (Figure 4.2A) and DNA (Figure 4.2B) for pellets cultured for 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. DNA levels increased steadily throughout the culture period but the mean levels of DNA at D7 and D14 were not statistically significant ($p>0.05$). GAG levels increased markedly throughout the culture period with statistical significance between the mean values at each time point ($p<0.05$).

**Morphology and immunolocalisation**

Standard histology confirmed that the pellets contained large amounts of ECM, as indicated by metachromatic staining with toluidine blue (Figure 4.3A). Immunolocalisation revealed extensive staining for type II collagen by day 14 (Figure 4.3B). Collagens type I & X staining failed for technical reasons and no further pellets were available for analysis.

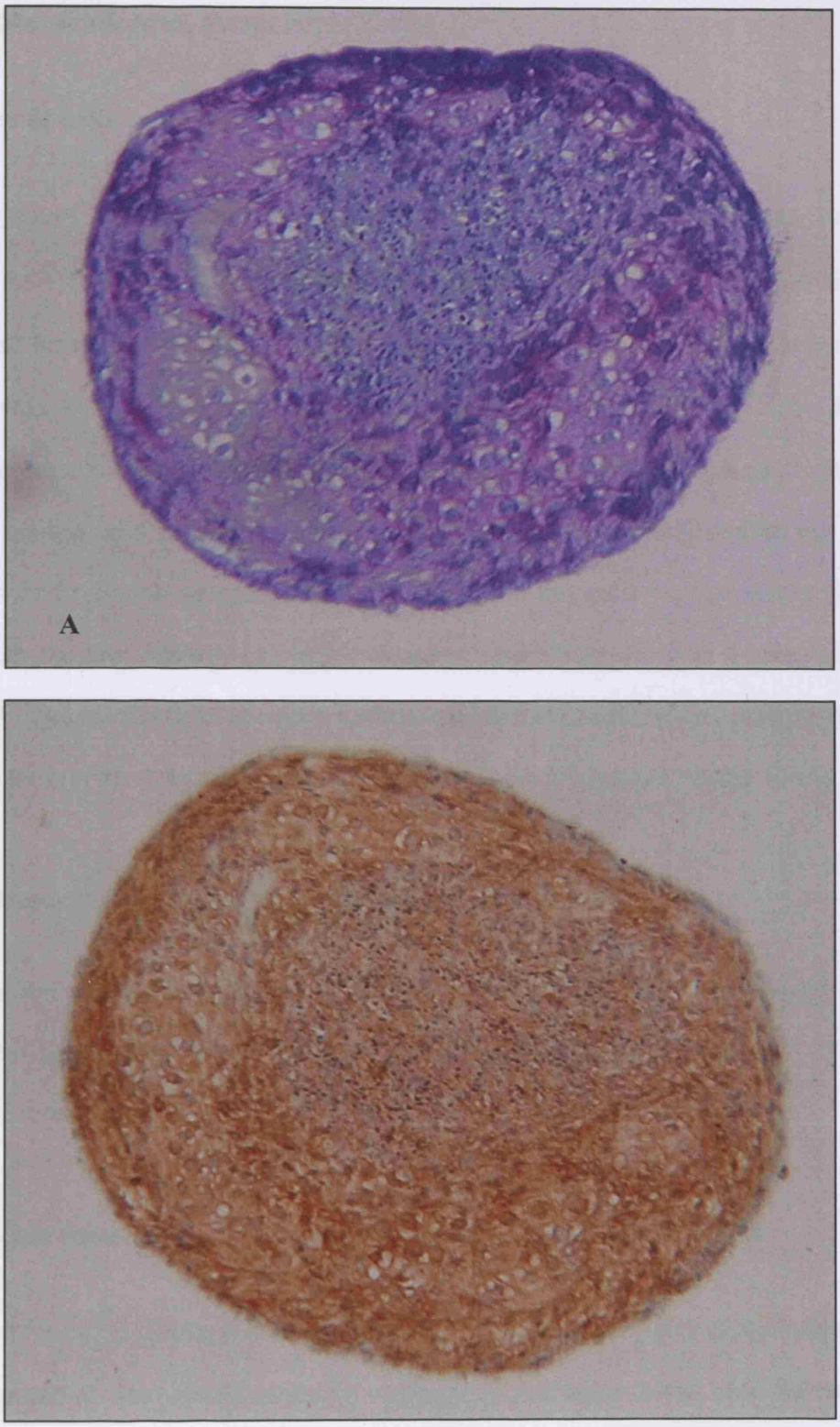



**Fig 4.3** Photomicrographs of pellet cultures prepared using human monolayer-expanded defect derived chondrocytes. The pellets were cultured for 14 days in DMEM/ITS+/TGFβ1 (A) toluidine blue stain (B) immunolocalisation for type II collagen. Magnification x132.

### 4.3.3 Mesenchymal Progenitor Cells

#### Number of cells isolated from bone marrow per patient

The amount of marrow obtained (±SE) varied between 5-20mL (average 12.54±1.14 (n=13)) with considerable variability between the number of cells obtained from each of the 13 patients. As indicated in Table 4.2, the mean value (±SE) was $2.96\pm0.61\times10^6$ cells.mL$^{-1}$ with a range of $1.07\text{-}7.50\times10^6$. Counting the cells accurately was difficult in view of the number of red cells present. Over the culture period and with subsequent media changes, cells adhered to the growth surface and became elongated and fibroblastic in appearance, as shown in Figure 4.4. With the first patient, cell isolation using only method 1 was employed, and it took 21 days for the cells to reach approximately 80% confluence. Using method 2, the mean time for the cells to reach confluence was 16.6 days (range 14-20).

#### Complications of Harvesting Procedure

Each of the patients from which a sample was harvested were re-examined one day after the procedure and apart from very minor, non painful bruising, none had complications.

#### Aggregate formation using human mesenchymal progenitor cells

After 48 hours no pellets were formed. However, within 96 hours of incubation, the cells formed an essentially spherical aggregate that became free from the walls of the tube and then became free floating in the DMEM/ITS+/Asc/TGF$\beta_1$ medium. No difference was evident between the ability of cells to pellet based on the isolation using either of the two methods.

**Table 4.2** Patient Details – mesenchymal progenitor cells (listed by patient age)

| Sample Number | Patient Age (years) | Sex | Amount of marrow obtained (mL) | Nucleated cell count | Cells $.mL^{-1}$ x $10^6$ | Time to First Passage (days) | Notes |
|---|---|---|---|---|---|---|---|
| 1 | 18 | M | 8 | $19x10^6$ | 2.38 | | infected |
| 2 | 19 | M | 15 | $40x10^6$ | 2.67 | 18 | |
| 3 | 25 | F | 20 | $40x10^6$ | 2.00 | 19 | |
| 4 | 25 | F | 15 | $16x10^6$ | 1.07 | 17 | infected |
| 5 | 26 | F | 15 | $111x10^6$ | 7.40 | 16 | mech loading |
| 6 | 28 | F | 8 | $60x10^6$ | 7.50 | 15 | |
| 7 | 30 | M | 12 | $13x10^6$ | 1.08 | 20 | |
| 8 | 30 | M | 15 | $50x10^6$ | 3.33 | 16 | mech loading |
| 9 | 30 | M | 10 | $18x10^6$ | 1.80 | 16 | |
| 10 | 31 | F | 15 | $16x10^6$ | 1.07 | 14 | |
| 11 | 38 | M | 15 | $38x10^6$ | 2.53 | 15 | |
| 12 | 41 | M | 10 | $12x10^6$ | 1.20 | 21 | Non Ficolled |
| 13 | 41 | M | 5 | $22x10^6$ | 4.40 | | infected |

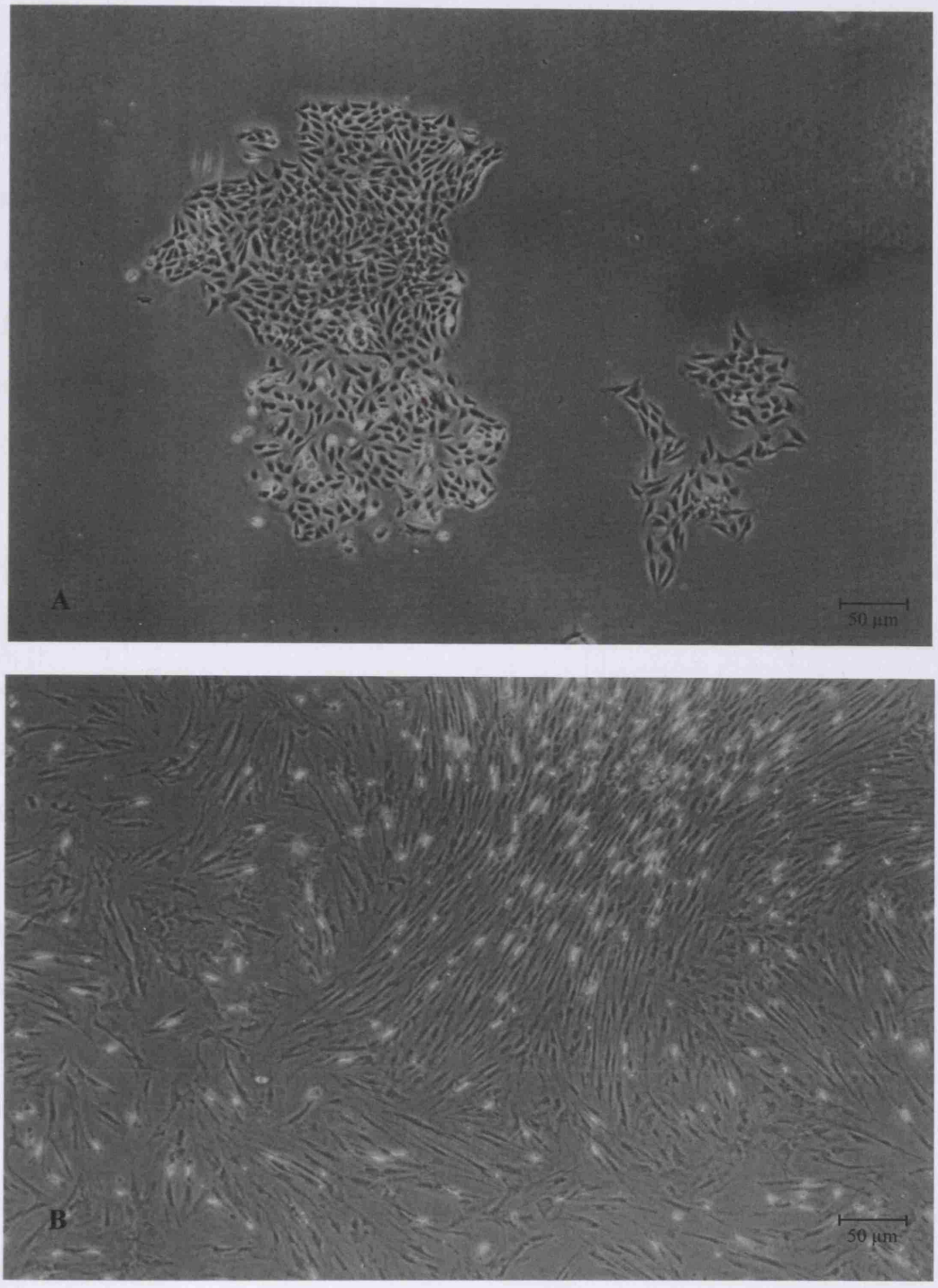



**Figure 4.4** Photograph of mesenchymal progenitor cells (A) after 7 days in culture in DMEM+10%FCS (note after 5 days the media was changed to remove the excess red blood cells) (B) after 20 days in culture.

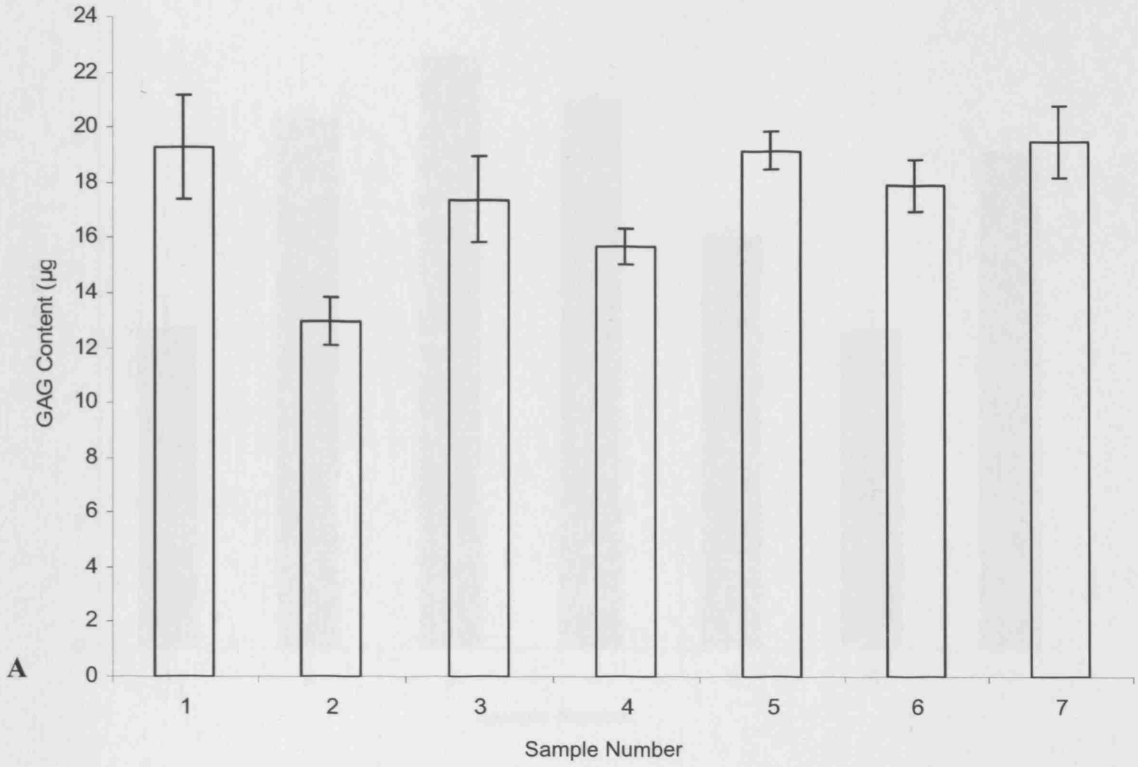
**Biochemical analysis**

Figure 4.5A illustrates the total GAG content from the human mesenchymal progenitor cells pellets of each of the respective patients after 14 days in culture in DMEM/ITS+/Asc/TGF$\beta_1$. Aside from sample 2 whose levels of GAG were lower, there was reasonable consistency of mean GAG content between patients. Figure 4.5B illustrates the total DNA content and Figure 4.5C illustrates the GAG/DNA for each of the respective samples. The DNA levels showed more marked variability, although GAG/DNA was consistent between 5.05-9.37.

Figure 4.6 illustrates total DNA (Figure 4.6A) and GAG (Figure 4.6B) for pellets cultured for 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. GAG levels increased throughout the culture period. There was no statistical significance between mean GAG values between day 4 and day 7 ($p>0.05$), however, mean GAG values at day 14 were significantly greater than day 7 ($P<0.05$). DNA levels decreased by day 7 in culture but then remained static for the remaining 7 days. The mean DNA value at day 7 was significantly lower than day 4 ($p>0.05$), however, mean DNA values at day 14 were not significantly different to values at day 4 ($P<0.05$).

**Morphology and immunolocalisation**

Standard histology confirmed that cells cultured in DMEM/ITS+/Asc/TGF$\beta_1$ displayed characteristics of chondrocytes by day 7, which increased in nature by day 14. The amount of ECM, as indicated by toluidine blue staining, increased mass creation with the amount of metachromatic staining (Figure 4.7). Immunolocalisation revealed staining for type II collagen increasing between days 7 and 14 (Figure 4.7 D-F). Type I staining was evident throughout the pellet during early culture although, by day 14, it was difficult to demonstrate (Figure 4.8B).



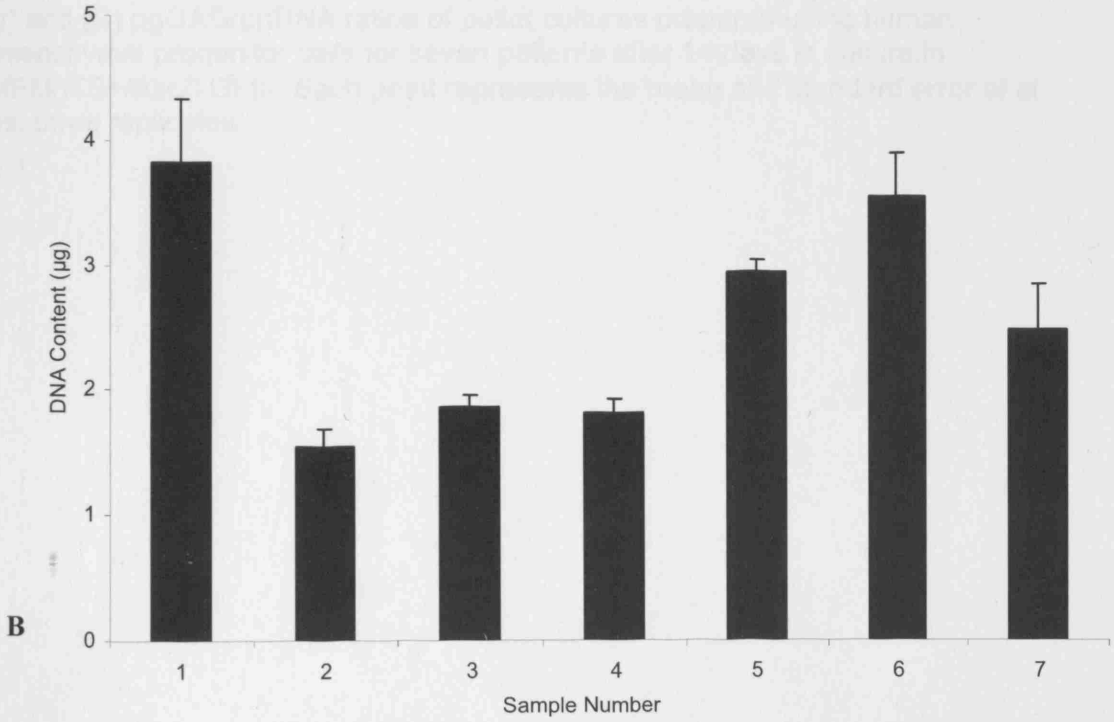




**Figure 4.5** Continued on next page

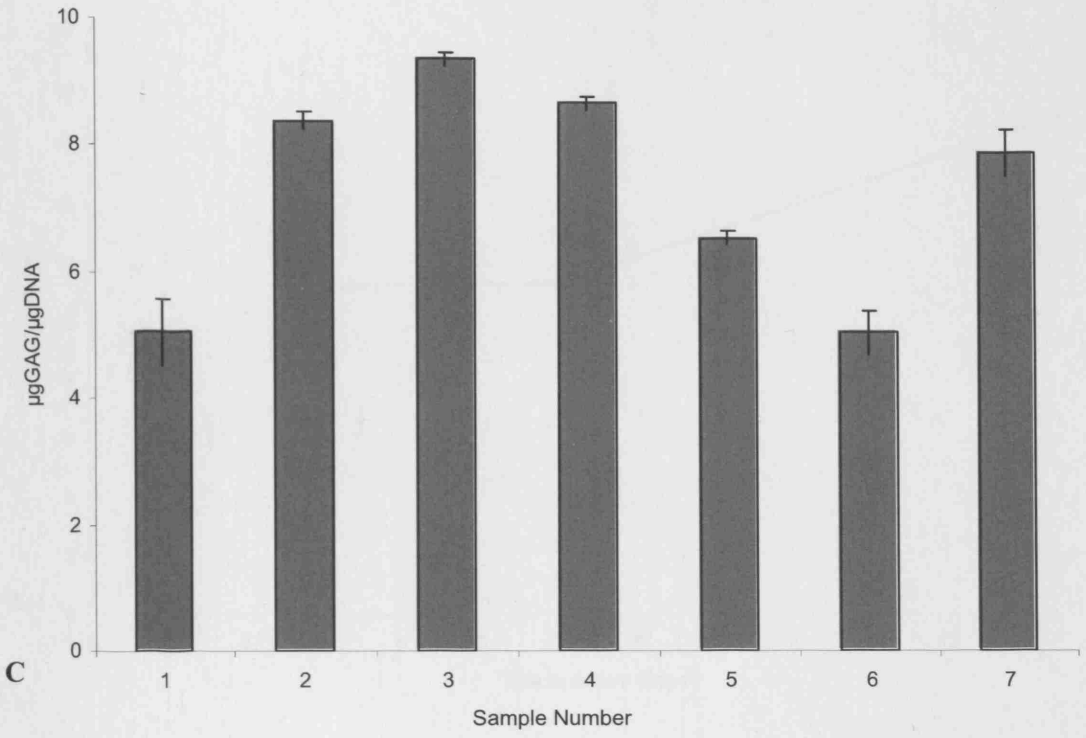



**Figure 4.5** Absolute Values (±SE) of (A) mean GAG content (µg), (B) DNA content (µg) and (C) µgGAG/µgDNA ratios of pellet cultures prepared using human mesenchymal progenitor cells for seven patients after 14 days in culture in DMEM/ITS+/Asc/TGF$\beta_1$. Each point represents the mean and standard error of at least three replicates.

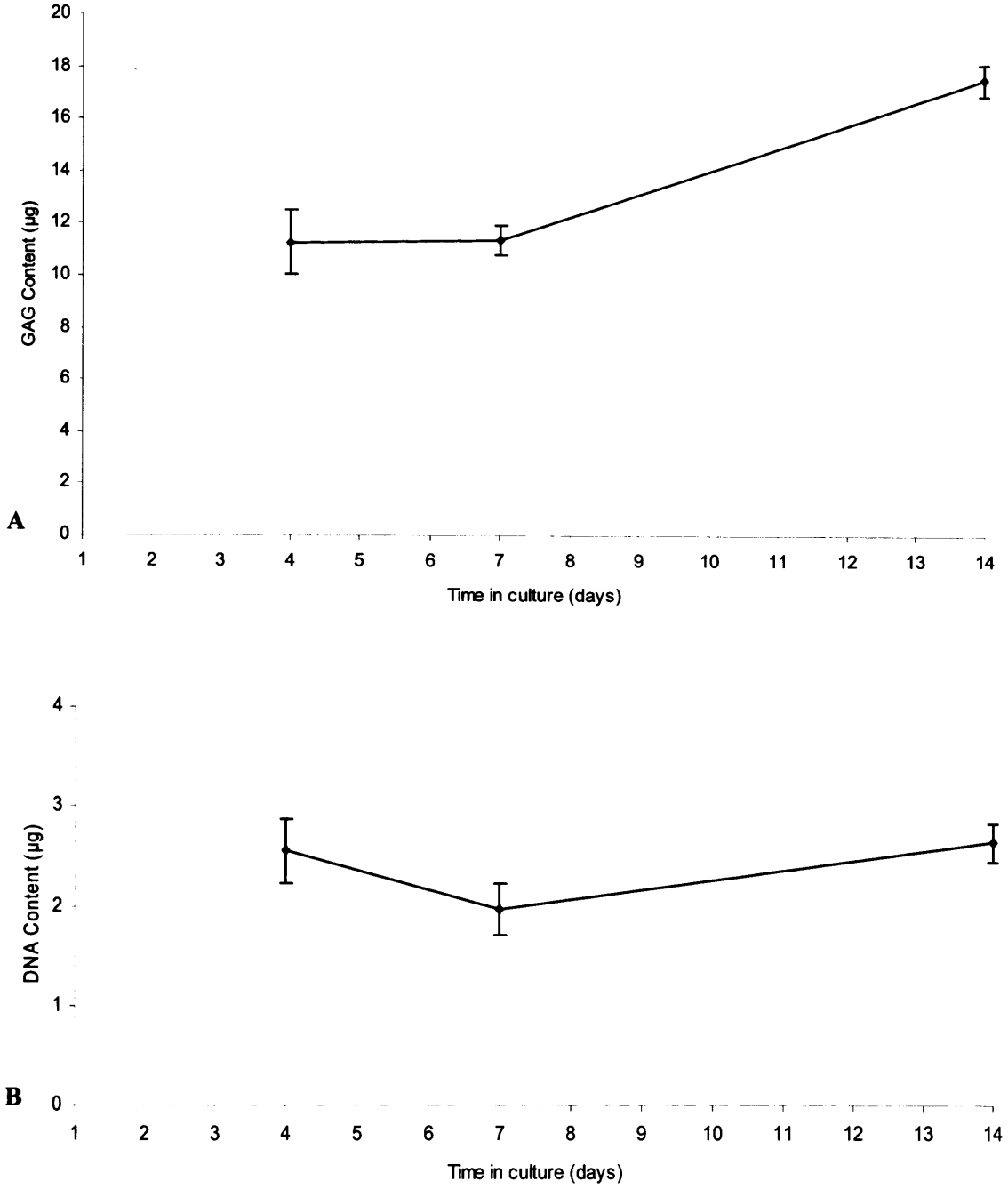



**Figure 4.6** GAG content (A) and DNA content (B) of pellet cultures prepared using human mesenchymal progenitor cells. The pellets were cultured for up to 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. Each point represents the mean and standard error of at least fourteen replicates from several separate experiments.

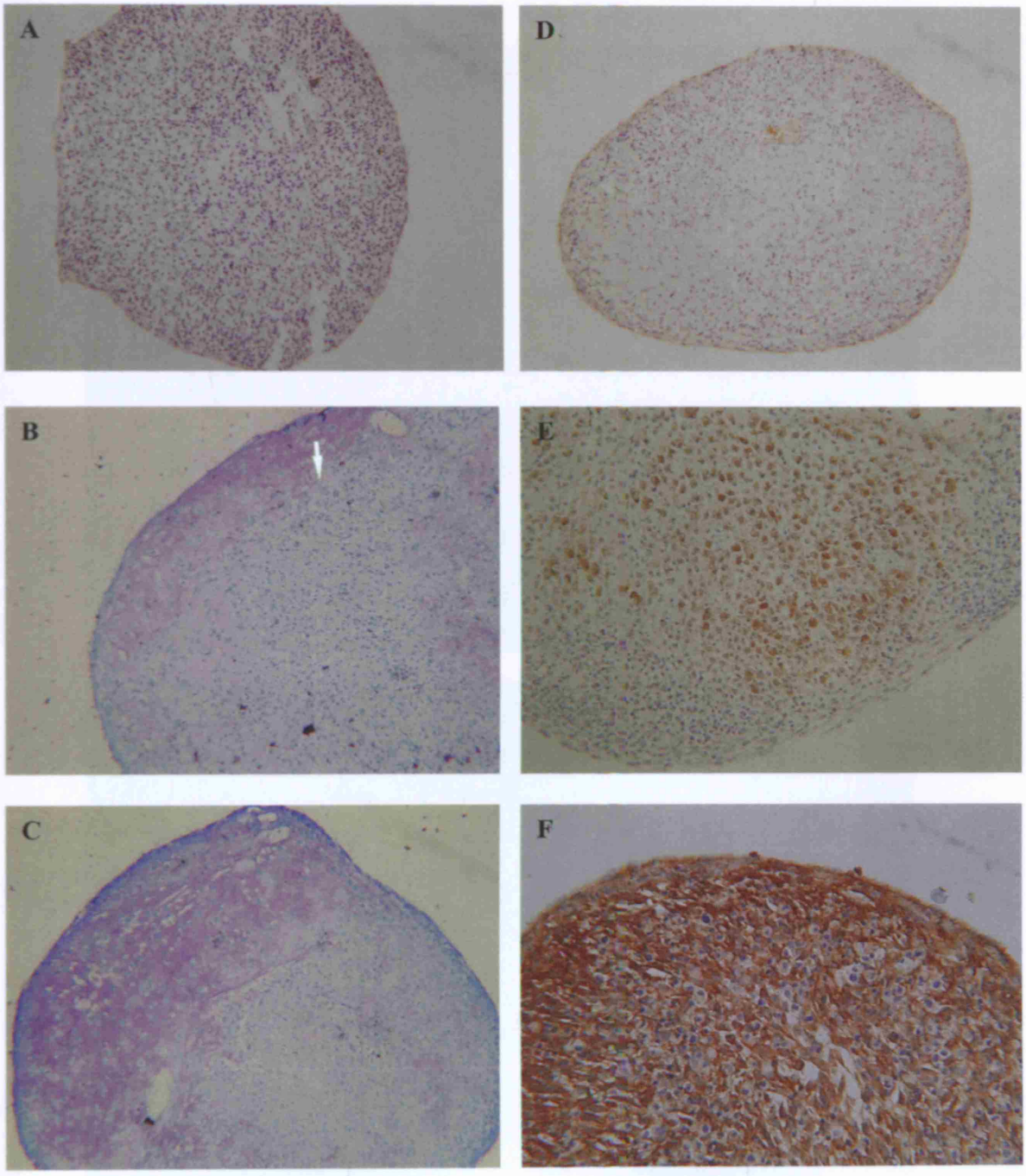



**Figure 4.7** Photomicrographs of pellet cultures prepared using human mesenchymal progenitor cells cultured for up to 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. (A&D) Day 2 (Magnification x96) (B&E) Day 7 (Magnification x132). (C&F) Day 14 (Magnification x132). (A-C) Toluidine Blue stain (D-F) Immunolocalisation to type II collagen.

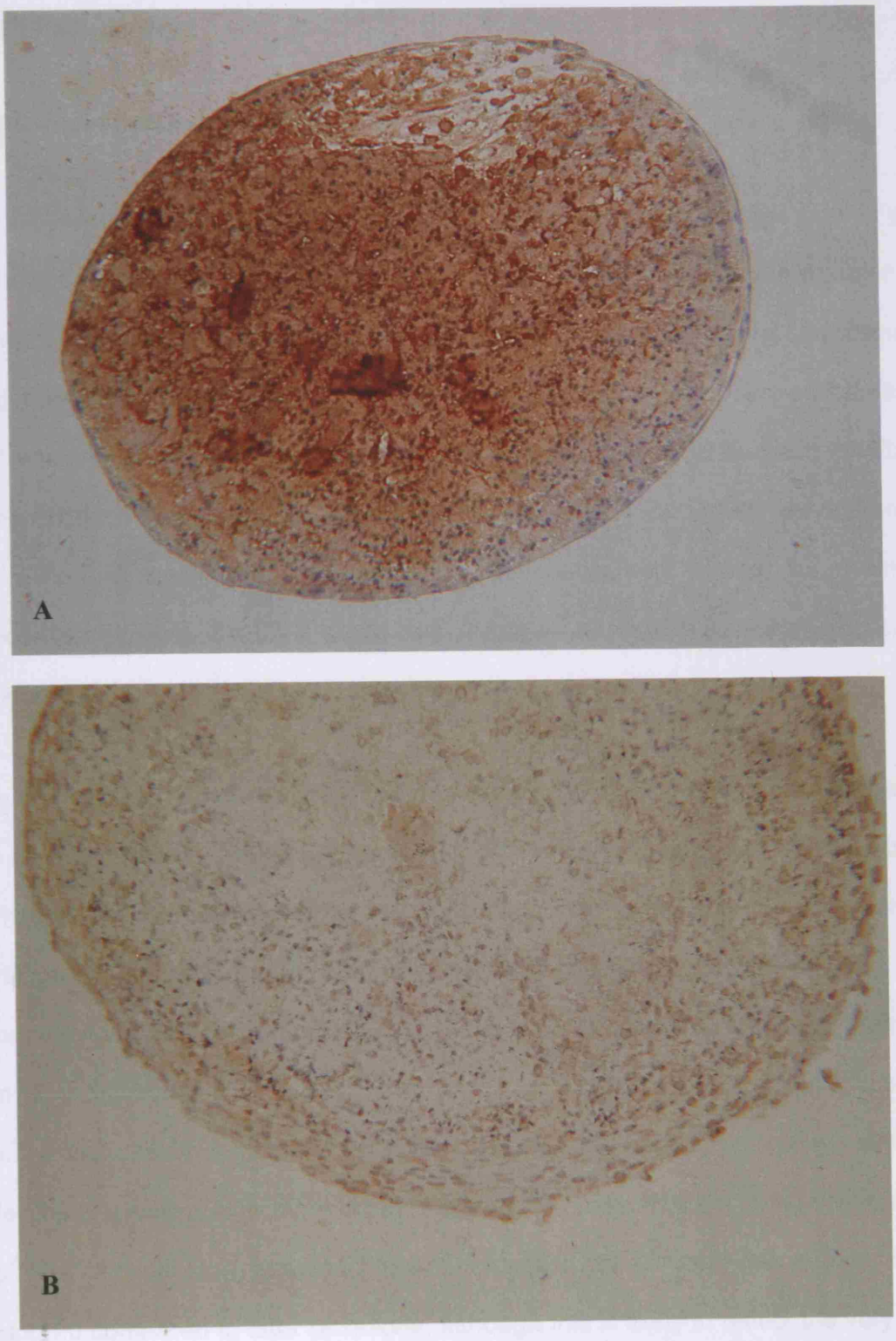



**Figure 4.8** Photomicrographs of pellet cultures prepared using human mesenchymal progenitor cells cultured for up to 14 days in DMEM/ITS+/Asc/TGF$\beta_1$. Immunolocalisation to type I collagen (A) Day 2 (Magnification x96) and (B) Day 14. (Magnification x 132).

## 4. 4 Discussion

**HDDC expanded in monolayer**

The process of sacrificing an area of healthy cartilage in order to repair another part of the knee is an unpleasant one for any surgeon. The concern lies in whether the harvesting procedure leads to any long term morbidity, such as painful symptoms or longer term the risk of developing osteoarthritis. Although, many of the advocates of ACI would say there is no evidence that the harvesting leads to ill effects (Brittberg *et al.,* 1994a; Peterson *et al.*, 2000; Lindahl *et al.,* 2001), due to the very nature of the procedure, the length of follow up and the assessment criteria, this is almost impossible to prove. Clearly it would be better if an alternative source of cells could be identified.

The author's experiments aimed to demonstrate that cartilage cored out as part of the second operative procedure contains viable chondrocytes, which when cultured in monolayer, behave in a similar manner to those harvested from a healthy area of cartilage. It was found that after initial seeding in monolayer (P0), the chondrocytes, derived from the defect, were slow to attach to the growth surface and typically retained a rounded morphology for a number of days. In the present study it took 10-14 days before the cells began to spread on the growth surface. After the first passage in monolayer, however, the cells attached and spread more rapidly. Cell expansion, assessed as an increase in cell number, was slightly higher during P0 compared to later passages, although this is likely to reflect the very long time to the initial passage. The doubling time was approximately 5 days in P0 and just over 2 days thereafter. These findings support previous work in the host laboratory (Wiseman *et al.*, 2004), in which bovine chondrocytes, in culture, demonstrated an initial lag phase during the first 7 days, followed by an increase in cell number thereafter. Indeed, these authors reported a 100 fold cell expansion

after 17 days in monolayer culture (Wiseman *et al.*, 2004). In the present work, a much slower cell expansion (55 times in 44 days) was found. This could reflect differences between species but, additionally, the batch of serum used could be a factor explaining the slow times found for cell expansion (Lennon *et al.*, 1996). In the process described by Brittberg medium supplemented with the patients own serum was used (Brittberg *et al.*, 1994b). This is not always the case in commercial processing of chondrocytes and many commercial suppliers of autologous chondrocytes use media supplemented with fetal calf serum, and this could, therefore, have important clinical consequences. The initial culture lag phase could also support the theory of articular cartilage progenitor cells (Dowthwaite *et al.*, 2004), in that only small numbers of progenitor cells might have been isolated from the damaged pieces of defect articular cartilage.

During monolayer culture the cells reverted to a fibroblastic morphology and on transference to a 3D environment the cells redifferentiated and produced abundant matrix including type II collagen. On histological analysis, no gross discernable differences were noted between the cell pellets produced using the cells from the defect and those cells obtained from healthy areas of the joint (chapter 3). These findings support previous work in which human chondrocytes from human adult knees were cultured for 4 weeks in type I collagen gels and demonstrated the synthesis of a cartilage-like matrix and the production of type II collagen (Chaipinyo *et al.*, 2004). However, in the present study the cells were cultured in a 3D medium after monolayer expansion and hence the findings are more reflective of the procedures used in the clinical practice of ACI.

Although harvesting the biopsy from the patients defect appears to be an attractive clinical alternative, there are also a number of theoretical and practical disadvantages. For example, the first stage biopsy is normally harvested

arthroscopically, whereas the process of debriding the cartilage defect takes place at a second sitting invariably as an open procedure. Assuming that the biopsy could be carried out arthroscopically during the first stage procedure, then this would likely leave the patient potentially debilitated for several weeks, whilst awaiting the second operative procedure. This might be addressed by the use of a fibrin patch/glue as a temporary filling in a similar manner to that used by dentists although this would have financial implications and would need further research to explore the feasibility.

In summary, the studies in this chapter revealed that the defect that is cored out from the patients knee prior to repair can be a viable source of cells for the clinical procedure of ACI and further exploration of this process is warranted.

**Mesenchymal Progenitor Cells**

Attempts of *in vivo* repair using mesenchymal progenitor cells have typically resulted in the formation of fibrocartilage as opposed to hyaline cartilage (Insall, 1967; Mankin, 1991; Meachim and Roberts, 1971; Shapiro *et al.*, 1993). Wakitani *et al*, implanted autologous mesenchymal progenitor cells in a collagen matrix into full thickness defects in rabbit knees (Wakitani *et al.*, 1994b). At 24 weeks they found repair of the subchondral bone with overlying cartilage tissue, although the tissue was more compliant than normal articular cartilage.

The development of an *in vitro* system to promote the chondrogenic potential of these cells allows for in depth study of the factors responsible for this complex process. Such a system has been used *in vitro* to differentiate mesenchymal cells of chickens (Quarto *et al.*, 1997), rats (Grigoriadis *et al.*, 1998), rabbits (Johnstone *et al.*, 1998) and humans (Yoo *et al.*, 1998; Mackay *et al.*, 1998).

Throughout the present studies, it has been shown that human mesenchymal progenitor cells can be isolated by self selection through monolayer culture and induced to chondrogenic differentiation using culture in a chemically defined serum-free medium. These results support the previous work involving human subjects (Yoo *et al.*, 1998). At the initiation of culture no chondrocytic markers were detected in the cell aggregates. Within a week, the presence of a metachromatic staining matrix, the chondrocytic appearance of the cells and the detection of type-II collagen on immunolabelling, demonstrated that the generated tissue was cartilaginous in nature. Previous studies have shown that attempts to initiate chondrogenesis from monolayer cultures under the same conditions were unsuccessful (Johnstone *et al.*, 1998). In this respect the cell aggregates appear to resemble the precartilage condensation of cells in the forming limb bud (Fell, 1925; Newman and Frisch, 1979).

In this study a plating density of $10x10^4$ cells per $cm^2$ was used, compared to a variable density in previous work (Wakitani *et al.*, 1994b; Yoo *et al.*, 1998; Johnstone *et al.*, 1998; Lennon *et al.*, 1996), which ranged from $5x10^3$ to $20x10^5$ per $cm^2$. The most appropriate cell density has not been identified and it is not known whether the cell density is an important variable. However, it is likely that a differential cell adhesion takes place whereby the appropriate cell lines adhere to the surface and hence self select from the population as a whole.

It has previously been described that the serum batch used for the culture medium is important to support the growth of adherent cells from marrow (Lennon *et al.*, 1996). In the current studies, the mean time between harvesting to the first cell passage was 16.6 days (range 14-20). A decision to passage was taken when the cells had reached approximately 80% confluence. Other studies have passaged at prescribed times, for example, 14 days independent of cell density (Johnstone *et*

*al.*, 1998), or between 10-14 days when the cells appeared to have reached confluence (Mackay *et al.*, 1998).

Ascorbic acid is thought to induce alkaline phosphatase and type X collagen, and has been shown to lead to calcium deposition in cultured chick chondrocytes (Shapiro *et al.*, 1991). Previous studies have shown that the cells in this system terminally differentiated into hypertrophic chondrocytes as indicated by changes in cell morphology and detection of type X collagens (Mackay *et al.,* 1998; Yoo *et al.*, 1998) and increased amounts of alkaline phosphatase activity (Johnstone *et al.*, 1998). The appearance of type X collagen is a rapid event occurring soon after the appearance of type II collagen. In the current studies, technical difficulties were encountered with the type X immunostaining and these data could not be verified.

Previous reports using human mesenchymal progenitor cells described pellets forming within 2 days (Yoo *et al.*, 1998). In the present work, pellets invariably took about 4 days to form. In chapter 3, dedifferentiated human passaged autologous chondrocytes were cultured in a similar serum free chondrogenic media and the cells always pelleted in less than 48 hours. This could reflect the serum batch used during the monolayer culture in a similar manner to that proposed previously, using human chondrocytes, namely, that the conditions of cell expansion can modulate their ability to re-enter the differentiation programme upon transfer into a 3D environment (Jakob *et al.*, 2001).

By day 14, the mean GAG content of the pellets had increased by 58% from the day 4 value, suggesting that the increase in pellet size was due to an accumulation of extracellular matrix rather than cell proliferation as DNA content did not increase accordingly. The matrix stained metachromatically to suggest the presence of

abundant proteoglycans and type II collagen staining was present throughout the pellet within 14 days (Figure 4.7).

Type I labelling was highest in the periphery coinciding with the cells with a flattened morphology and this supports previous findings (Mackay *et al.,* 1998; Yoo *et al.*, 1998; Johnstone *et al.*, 1998). The overlap of staining patterns of type II and type I collagens may reflect a gradual change from fibroblastic to chondrocytic phenotype (Mackay *et al.*, 1998). Pellet culture may indeed provide a microenvironment for mesenchymal progenitor cells that promotes chondrogenic differentiation in the centre of pellets, while remaining amenable to fibroblastic or synovial differentiation near the periphery. Alternatively, given the heterogeneous nature of the cell origins, these cells might in fact have been derived from a separate fibroblastic-type phenotype and that a cell sorting phenomenon occurred during the formation of the cell aggregates.

Different isoforms of the growth factor have been previously used, for example, $TGF\beta_3$ (Mackay *et al.*, 1998) or $TGF\beta_1$ (Johnstone *et al.*, 1998; Yoo *et al.*, 1998). In the current study $TGF\beta_1$ was used and no comparative assessment of TGFβ isoforms was attempted.

In this study a technique for harvesting bone marrow has been identified, that is rapid and easy to perform and presented no complications to the author. Additionally the chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells appears to be retained through multiple passages as has been described previously (Yoo *et al.,* 1998). This clearly represents an important attribute if these cells are to be multiplied initially and used for repair techniques in clinical practice. Indeed, this approach could replace the need for a biopsy of healthy cartilage in the first stage of the cartilage replacement procedure.

In this situation, the patient would have a bone marrow aspiration performed, possibly under local anaesthetic, four weeks prior to the definitive operative procedure. The technique can provide unlimited amounts of cells for transplantation and this is a promising area which deserves further research.

# CHAPTER 5 – MECHANICAL STIMULATION OF CELLS

## 5.1 Introduction

In chapter three the modulation of chondrocytes using chemical stimulation in a controlled environment was explored. *In vivo* the processes that modulate chondrocytes are however complex. During normal walking, articular cartilage is subjected to dynamic loading, applied perpendicular to the articular surface and this is known to alter the metabolism of the embedded chondrocytes. Molecular events regulating responses of chondrocytes to mechanical forces are increasingly examined, and are thought to be mediated via an integrin-dependent interleukin (IL)-4 autocrine/paracrine loop (Salter *et al.,* 2002; Salter *et al.*, 2001).

Normal physiological loads compress the cartilage by a strain of up to 30% (Guilak, 1994; Guilak, 2000). There is evidence that a persistent decrease in joint loading or immobilisation of the joint can lead to a decrease in the concentration of proteoglycans and alterations in the mechanical properties of the cartilage. Thus maintenance of the normal composition of articular cartilage requires a minimal level of loading and motion of the joint (Buckwalter, 1995).

Previous *in vitro* studies have shown that static compression can inhibit proteoglycan synthesis (Saamanen *et al.*, 1990; Kim *et al.*, 1994), whereas dynamic compression applied to cartilage explants or isolated chondrocytes within 3D constructs has the ability to enhance cellular activity in a frequency dependant manner (Sah *et al.,* 1989a; Buschmann *et al.*, 1995; Lee and Bader 1997).

Cells used for tissue engineered cartilage repair systems are typically isolated from a low load-bearing site at the periphery of the cartilaginous joint surface and

expanded in monolayer prior to re-implantation and, as confirmed in chapter three, become dedifferentiated in the process. Two key issues arising from this have yet to receive much attention. First do harvested cells from a low-load bearing area respond differently to mechanical load than those from a high-load bearing area and, secondly, does the phenotypic modulation of chondrocytes following passage in monolayer influence specific mechanotransduction processes and thereby alter the response of the cells to the application of mechanical conditioning regimes. The majority of studies investigating the influence of loading on chondrocytes within 3-D constructs have used bovine cells. In addition the presence of compounding variables such as tissue age, scaffold material and load regime renders definitive conclusions regarding the effect of passage on mechanotransduction processes difficult to interpret. Accordingly the present study was designed to assess the response of passaged human articular chondrocytes used in clinical practice, to dynamic compression.

In addition, as demonstrated in chapter 4, mesenchymal progenitor cells display the ability to differentiate along the chondrogenic lineage through modulation using chemical stimuli. *In vivo* controlled mechanical stimulation has been shown in a rat model to induce cartilage from mesenchymal tissue (Tägil and Aspenberg, 1999) and *in vitro*, mechanical loading in the presence of a chemically defined medium supplemented with growth factors has been shown to stimulate chondrogenesis (Mauck *et al.*, 2003; Angele *et al.*, 2003; Huang *et al.*, 2004).

The present study was performed to assess whether mechanical loading can induce undifferentiated human mesenchymal progenitor cells to proceed along the chondrogenic lineage in the absence of chemical stimulation.

## 5.2 Materials & Methods

A specially designed cell-straining apparatus (Dartec, Stourbridge, U.K) was employed (Lee and Bader, 1995b). The loading frame contained a vertical assembly whose movement was monitored with a linear variable displacement transducer (model M5/1000; RDP Electronics, Wolverhampton, U.K) and incorporated a standard load cell (range 0-2,000 lb [907.2kg]) (model 41/57/P/0; Sensotec, Wolverhampton. U.K). The vertical assembly entered the tissue incubator (Heraeus Instruments, Brentwood, U.K) through a hole where it was designed to be attached to a central rod that, in turn, was attached to a mounting plate located within a Perspex (methylmethacrylate) box. The vertical movement of the central rod relative to the box was prevented by means of a locking screw.

A fixed rod entered the incubator through a similarly located hole in the base of the incubator and was attached to a circular platen. The mounting plate was divided into five sections held together by a series of clamping nuts and locating rods. The assembled plate incorporated a matrix of 24 holes, the innermost of which constrained the vertical movement of 12 loading pins (4 mm in diameter, 31 mm in length, and 2 g in weight), while the outermost allow free movement of 12 identical pins. The pins incorporated an 11 mm circular Perspex indenter.

During the assembly process, the outermost pins were held in a fixed position by removable plastic collars. The Perspex box, including the central rod, mounting plate, and loading pin arrangement, was sterilized in 100% methyl alcohol and placed in a sterile environment before the experiments were set up. The central rod was locked to prevent vertical movement during all preliminary procedures as illustrated in Figure 5.1.

### 5.2.1 Isolation of Passaged Human Autologous Chondrocytes

Cartilage biopsies were harvested from the medial femoral condyles of 7 patients, mean age 28 years (range 18-40) undergoing ACI. Details of these seven patients are listed in Table 5.1. The cells were expanded in monolayer culture using medium supplemented with autologous serum, in a technique proprietary to Verigen Transplantation Services for approximately 4 to 5 passages. Following expansion, the cell number and viability of a proportion of the cells from each patient was assessed at the supply laboratory using the trypan blue exclusion dye test and the cells were subsequently couriered by overnight delivery in a proprietary transport medium (Verigen Transplantation Service International, Leverkeusen, Germany). At the test laboratory, cells deemed surplus to the requirements of the ACI procedures were taken to the laboratory within one hour for processing. In the laboratory the cells were resuspended in DMEM+10%FCS and counted under phase contrast as described earlier.

During the time of study, three further cartilage samples were available that were processed in the laboratories at the Institute of Orthopaedics, Stanmore, UK. The first case was a 21-year-old man undergoing fusion of the knee for unremitting pain following a fracture (Patient 8). The proximal tibia and distal femoral articular surfaces were obtained directly from the theatre and transported in normal saline in a sterile container and taken immediately to the laboratory for processing. The second sample was cartilage taken from the defect of a 36-year-old male patient with a 2x1cm osteochondral defect of his right medial femoral condyle (Patient 9). The third was a 15-year-old male, who had undergone an above knee amputation for a bone malignancy that did not extend to the articular surface (Patient 10). The sample had been previously handled by the histopathologist in normal processing,

although the joint had not been opened prior to the sample being made available for further cellular analysis.

Where appropriate and under sterile conditions, the cartilage was removed from the subchondral bone using a size 15 scalpel and cells were isolated using the technique described previously. Following cell isolation, the cells were washed in EBSS several times, resuspended in DMEM+10%FCS and counted using the Trypan blue exclusion test as described previously (Fong and Kissmeyer-Nielsen, 1972). The cells from Patient 8 (Table 5.1) were used as fresh isolates for the purposes of the loading experiment. The cells from Patient 9 were plated into a 75cm$^2$ flask and cultured in monolayer in DMEM+10%FCS at 37°C/5%$CO_2$ for 4 passages as described previously. On the 4$^{th}$ passage the cells were counted using the trypan blue dye test (Fong and Kissmeyer-Nielsen, 1972) in preparation for the mechanical loading experiments.

### 5.2.2 Isolation of mesenchymal progenitor cells

Bone marrow samples were obtained from the posterior iliac crests of two patients as described earlier. One patient was a 26-year-old female and one a 30-year-old male (see Table 5.2). Mesenchymal progenitor cells were isolated using the Ficoll®-Paque Technique (Amersham Pharmacia Biotech), and cultured in monolayer for four passages as described earlier. On the 4$^{th}$ passage the cells were resuspended in 10mL DMEM+10%FCS and counted using the trypan blue test.

### 5.2.3 Preparation of the agarose-chondrocyte cylinders

Cell numbers and viability were assessed using the trypan blue dye exclusion test. The cells were then resuspended at $8x10^6$ cells.mL$^{-1}$. The chondrocyte or mesenchymal progenitor cell suspension was then added to an equal volume of 6%

agarose (type VII, Sigma Chemicals) in Earle's balanced salt solution to give a final concentration of $4x10^6$ cells.$mL^{-1}$ in 3% agarose.

The mixed agarose-cell suspension was plated in specially designed Perspex molds and allowed to gel at 4°C for 20 minutes. Cylindrical constructs (5 mm in diameter and 5 mm in height) were created. The cylinders were cultured in 1mL of Dulbecco's minimal essential medium with 10%FCS in a 24-well plate (Costar, High Wycombe, U.K) at 37°C in 5% $CO_2$ for 16 hours to allow equilibration to culture conditions.

Twenty-four identical agarose-cell cylinders were centrally located in each well of a 24-well tissue culture plate after removal of the surrounding medium. The plate was then positioned within the sterile Perspex box, such that each cylinder was immediately beneath a corresponding Perspex indenter of the protruding loading pins. The pins were unclamped and each was carefully lowered onto the cylinder to ensure contact between the pin and the agarose (Figure 5.1). Thus, the mass of the loading pin alone was applied to each agarose-cell cylinder. The tangent modulus of the agarose-chondrocyte system was previously estimated from the linear region of its response in unconfined compression as 120kPa (Lee and Bader, 1995b; Knight *et al.*, 1996). Therefore, an equilibrium tare strain of approximately 0.8% was applied to each agarose-cell cylinder.

The innermost 12 pins were then clamped to prevent vertical movement in relation to the mounting plate. One millilitre of DMEM+10%FCS + 10μCi.$mL^{-1}$ + $^{35}SO_4$ + 1μCi.$mL^{-1}$ [$^3$H]thymidine (37,000 Bq.$mL^{-1}$, Amersham International, Amersham, U.K) was introduced into each well using a right-angled needle and syringe. The box was then closed, transferred from the hood to the incubator and placed on the base platen. The crosshead was moved slowly downward and was attached to the

central rod, which was subsequently unlocked to allow free vertical movement. In this position, the cylinders were considered to be subjected to the tare strain. The control electronics were set to produce a cross head movement equivalent to maximum strain amplitude of 15% with a range between 0-15%. A sinusoidal waveform was used at a 1Hz frequency, as described previously (Lee *et al.*, 1998). The innermost plugs acted as experimentally strained specimens, while the outermost plugs acted as unstrained controls. All cell/agarose constructs were incubated at 37°C/5%$CO_2$ for 48 hours.

At the end of the culture period, the medium was removed and was stored at –20°C prior to glycosaminoglycan analysis as described previously (Section 3.2.2). The cylinders were incubated at 70°C in 1mL phosphate buffered saline, supplemented with 10mM EDTA and 10mM cysteine HCl until molten. The suspension was cooled to 40°C, and 5µL of a papain suspension containing 3IU papain and 10IU agarase (both Sigma Chemical) was added. The solution was incubated for 24 hours at 40°C.

**Table 5.1** Details of the patients whose cell samples underwent mechanical loading.

| Sample Number | Patient Age | Diagnosis |
|---|---|---|
| 1 | 18 | 2.5 x 1cm MFC OCD |
| 2 | 19 | 2.3 x 2cm MFC OCD |
| 3 | 23 | 1.5 x 1cm MFC OCD |
| 4 | 31 | 1 x 1.5cm Lateral femoral condyle OCD |
| 5 | 33 | 1 x 1cm Medial facet patella OCD |
| 6 | 34 | 1.4 x 1.2cm Med facet patella OCD |
| 7 | 40 | 2.5 x 0.8cm MFC OCD |
| 8 | 21 | Arthrodesis following trauma |
| 9 | 36 | Cells from a 2x1cm MFC OCD |

*Note patients 1-7 were autologous chondrocytes processed in the laboratories of Verigen in Germany and are listed in order of ascending age; patient 8 was freshly isolated chondrocytes, processed in the test laboratory and patient 9 was passaged cells from the defect cultured in DMEM+10%FCS for four passages. Cells were available from one further patient, aged 15, following total knee joint excision for malignancy. The cells from this patient became infected, however, during early culture, and were discarded. Note that MFC=Medical Femoral condyle and OCD=osteochondral defect.*

**Table 5.2** Details of the patients, from which mesenchymal progenitor cells were obtained.

| Sample Number | Patient Age | Diagnosis |
|---|---|---|
| 10 | 26 | Mesenchymal Stem Cells at Passage 4 (Female) |
| 11 | 30 | Mesenchymal Stem Cells at Passage 4 (Male) |

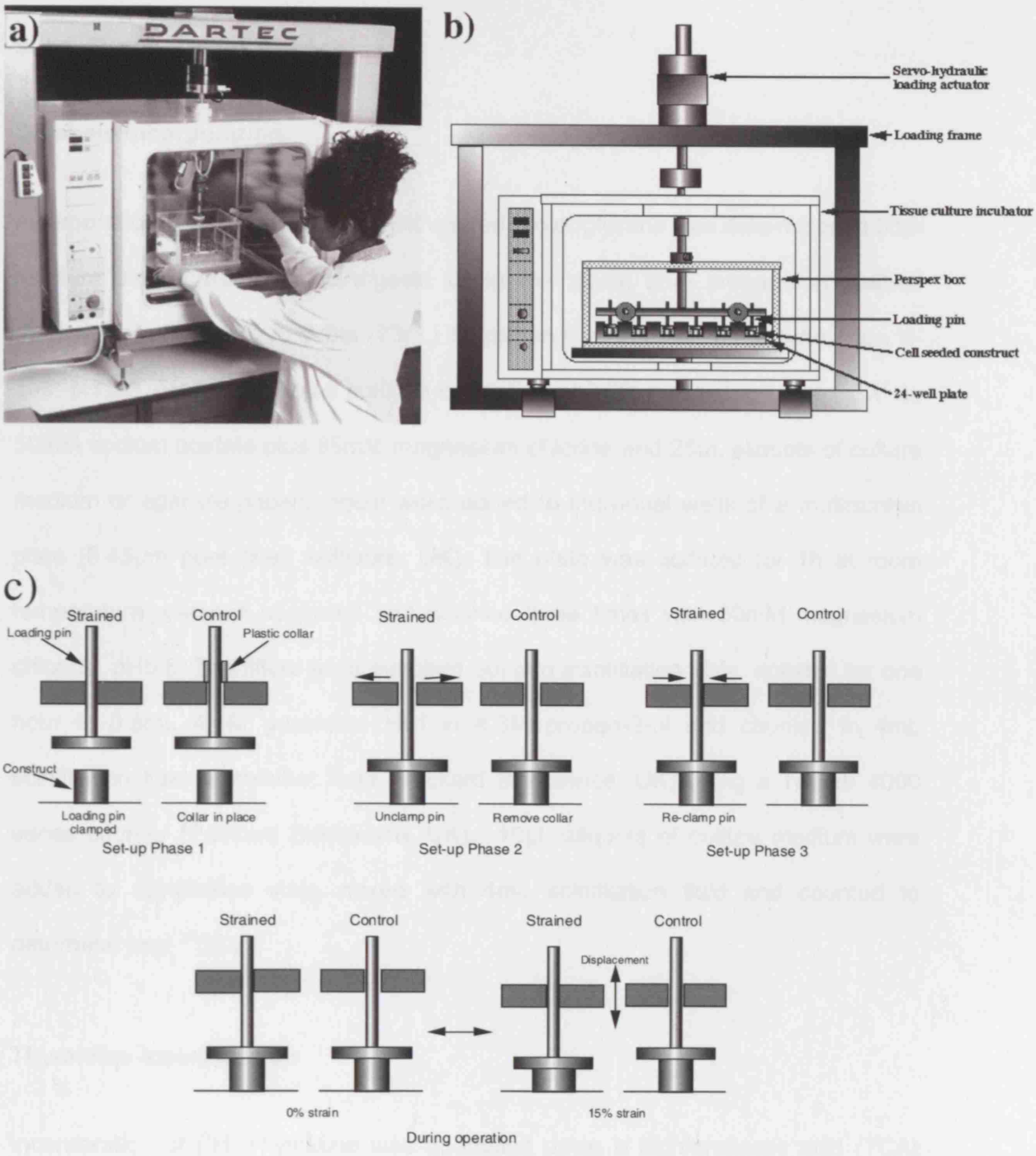



**Figure 5.1** Photograph (a) and schematic representation (b) of the Compressive cell strain bioreactor. (c) Schematic representation of the system during set-up and in operation (adapted from Shelton *et al.*, 2003).

### 5.2.4 Biochemical Assessment

#### Sulphate Incorporation

Incorporation of $^{35}SO_4$ into newly synthesized proteoglycans was determined in both medium and agarase/papain digests using the alcian blue preparation method (Masuda *et al.*, 1994). Aliquots (75µL) 50mM sodium acetate, 0.5% (v/v) triton X-100, pH 5.8, 50µL alcian blue solution consisting of 0.2% (w/v) alcian blue 8GX$^2$ in 50mM sodium acetate plus 85mM magnesium chloride and 25µL aliquots of culture medium or agarase/papain digest were added to individual wells of a multiscreen plate (0.45µm pore filter, Millipore, UK). The plate was agitated for 1h at room temperature, vacuum aspirated and washed three times with 50mM magnesium chloride, pH5.8. The filters were punched out into scintillation vials, agitated for one hour in 0.5mL 4mM guanidine HCl in 4.3M propan-2-ol and counted in 4mL scintillation fluid (Emulsifier Safe, Packard Bioscience, UK) using a Tricarb 4000 series counter (Packard Bioscience, UK). 10µL aliquots of culture medium were added to scintillation vials, mixed with 4mL scintillation fluid and counted to determine total $^{35}SO_4$.

#### Thymidine Incorporation

Incorporation of [$^3$H]-Thymidine was quantified using a trichloroacetic acid (TCA) precipitation technique. One hundred microlitres of papain/agarase digest was added to individual wells of a multiscreen plate (0.45µm pore filter, Millipore, Watford, UK). 100µL of 20% (w/v) TCA was added to each well and incubated at 40°C for 30 minutes. The multiscreen plate was vacuum aspirated and 100µL 10% (w/v) TCA was added to each well. The plate was vacuum aspirated, dried and the filters were punched out into scintillation vials. The filters were agitated for 30

minutes in 0.5μL 0.05mM KOH to release bound tritiated thymidine into solution and counted in 4mL scintillation fluid (Emulsifier plus, Packard Bioscience, Pangbourne, UK) using the Tricarb 4000 series counter.

DNA concentrations in the constructs were determined using the bisbenzamide Hoescht H33258 method (Rao and Otto, 1992) as described previously. $^{35}SO_4$ and [$^3$H]-Thymidine incorporation were expressed per μg DNA.

### 5.2.5 Determination of chondrocyte viability

In preliminary experiments fluorescence microscopy was carried out after 48 hours of loading to assess cell viability using the fluorescein live/dead stain. Sample agarose-chondrocyte constructs, selected in a random manner, were stained with Cacein AM and Ethidium Homodimer (both Molecular Probes, Eugene, USA). Staining was performed over one hour at 37°C by the addition of 5μL of each stain to 5mL of DMEM+10%FCS. After one hour, the constructs were cut in half and the cut surface placed flush against a cover slip. The cut surface was examined with a either a red, green or a mixture of both filters using a fluorescence microscope (Olympus, London, UK) through the cover slip.

### 5.2.6 Statistical Analysis

The Student's t-test was used to examine differences between ratio values and the corresponding values for unstrained controls. A level of statistical significance was set at 5 per cent ($p<0.05$).

## 5.3 Results

### Noted exclusions

The cells from Patient 10 became infected very early on in the culture process and had to be discarded in entirety. Two cell-agarose composites were crushed during two experiments. In both cases the samples were excluded from analysis. Accordingly, 6 replicate samples and 6 controls were available for each patient.

### 5.3.1 Cell Viability

After 48 hours, both viable and non viable chondrocytes could be seen under the fluorescent microscope (see Figure 5.2). The vast majority of cells were viable as indicated by green staining. A smaller but widely distributed number were identified as being dead and stained red. It was not possible to estimate the percentage cell viability accurately due to the lack of image clarity of the specimen with a thick cross sectional area.

### 5.3.2 Biochemical Results

#### Sulphate & Thymidine Incorporation Data

#### Chondrocytes expanded in medium supplemented with autologous serum

Absolute $^{35}SO_4$ incorporation by unstrained chondrocytes for each of the patients is illustrated in samples 1-7 in Figure 5.3. Clearly there was variability in absolute $^{35}SO_4$ incorporation data from the cells of the seven patients. The samples have been displayed in order of ascending patient age. Samples 1-7 from Figure 5.4 indicates the absolute values of [$^3$H]-thymidine incorporation by unstrained chondrocytes. A similar trend was observed to the results of $^{35}SO_4$ incorporation

The effects of dynamic compression on $^{35}SO_4$ incorporation are shown in Figure 5.5. It revealed that in 6 out of 7 cases the mean ratio of loaded to unloaded values was consistently below 1.0. This indicated a compression-induced inhibition in $^{35}SO_4$ incorporation, with a maximum reduction of 32% (patient 2). By contrast, the sample corresponding to patient 6 revealed a statistically significant stimulation of $^{35}SO_4$ incorporation ($p<0.05$).

The effects of dynamic compression on [$^3$H]-thymidine incorporation are shown in Figure 5.6. The normalized means of the loaded data reveal an inhibition of [$^3$H]-thymidine incorporation in 5 out of 7 cases, with a maximum value of 19% (patient 6). By contrast, two samples (patients 1 and 4) revealed a small stimulation in thymidine incorporation ($p>0.05$).

**Chondrocytes expanded in medium supplemented with fetal calf serum**

Freshly isolated autologous chondrocytes, from a young patients knee (Patient 8) and passaged chondrocytes from the defect (Patient 9) revealed similar absolute data for $^{35}SO_4$ incorporation than for samples 1-7, as demonstrated in Figure 5.3. [$^3$H]-thymidine incorporation, showed variation between the two patients as shown in Figure 5.4 but also was of a similar order of magnitude as that for samples 1-7. The passaged cells from the defect (sample 9) displayed greater [$^3$H]-thymidine incorporation than the freshly isolated cells of sample 8.

The effects of dynamic compression on $^{35}SO_4$ incorporation on the cells of patients 8 and 9 are shown in Figure 5.5. The normalized means of both the freshly isolated cells (patient 8) and the passaged cells (patient 9) were both significantly reduced indicating an inhibition of metabolism in response to loading ($p<0.05$).

The effects of dynamic compression on [$^3$H]-thymidine incorporation on samples 8 and 9 are shown in Figure 5.6. The passaged cells from sample 9 revealed an inhibition of [$^3$H]-thymidine incorporation whereas [$^3$H]-thymidine incorporation was stimulated significantly in the freshly isolated cells of sample 8 ($p<0.05$).

**Mesenchymal Progenitor Cells**

The absolute $^{35}SO_4$ incorporation data from the mesenchymal progenitor cells of patients 10 and 11 is shown in Figure 5.3 and proliferation as measured by [$^3$H]-thymidine incorporation is demonstrated in the two patients (sample 10 and 11) in Figure 5.4.

The effects of dynamic compression on $^{35}SO_4$ incorporation on the cells of patients 10 and 11 are shown in Figure 5.5. The normalized means of the $^{35}SO_4$ incorporation revealed stimulation of matrix metabolism in both patients. Only in patient 11 was this increase statistically significant ($p<0.05$).

The effects of dynamic compression on [$^3$H]-thymidine incorporation on samples 10 and 11 are shown in Figure 5.6. The normalized means revealed stimulation in both samples of statistical significance ($p<0.05$).

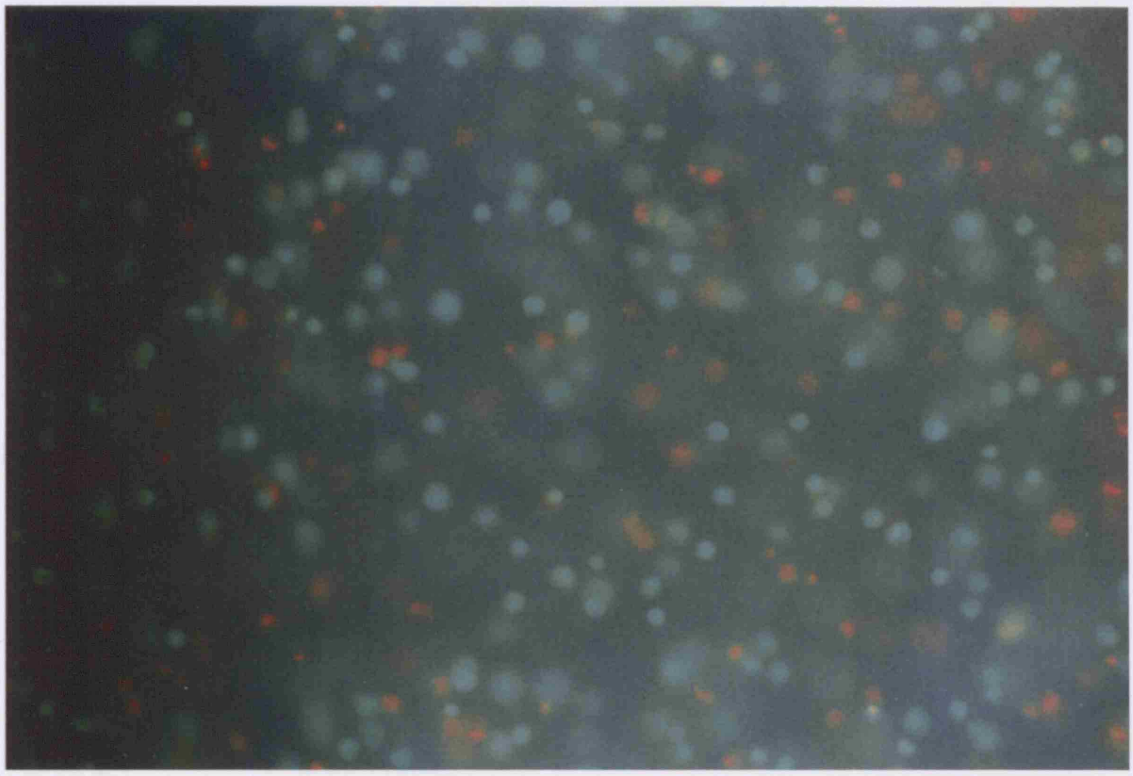


**Figure 5.2** Photomicrograph of an agarose-chondrocyte construct stained with Calcein AM and Ethidium fluorescein live/dead stain. Note the green cells are alive and red cells dead.

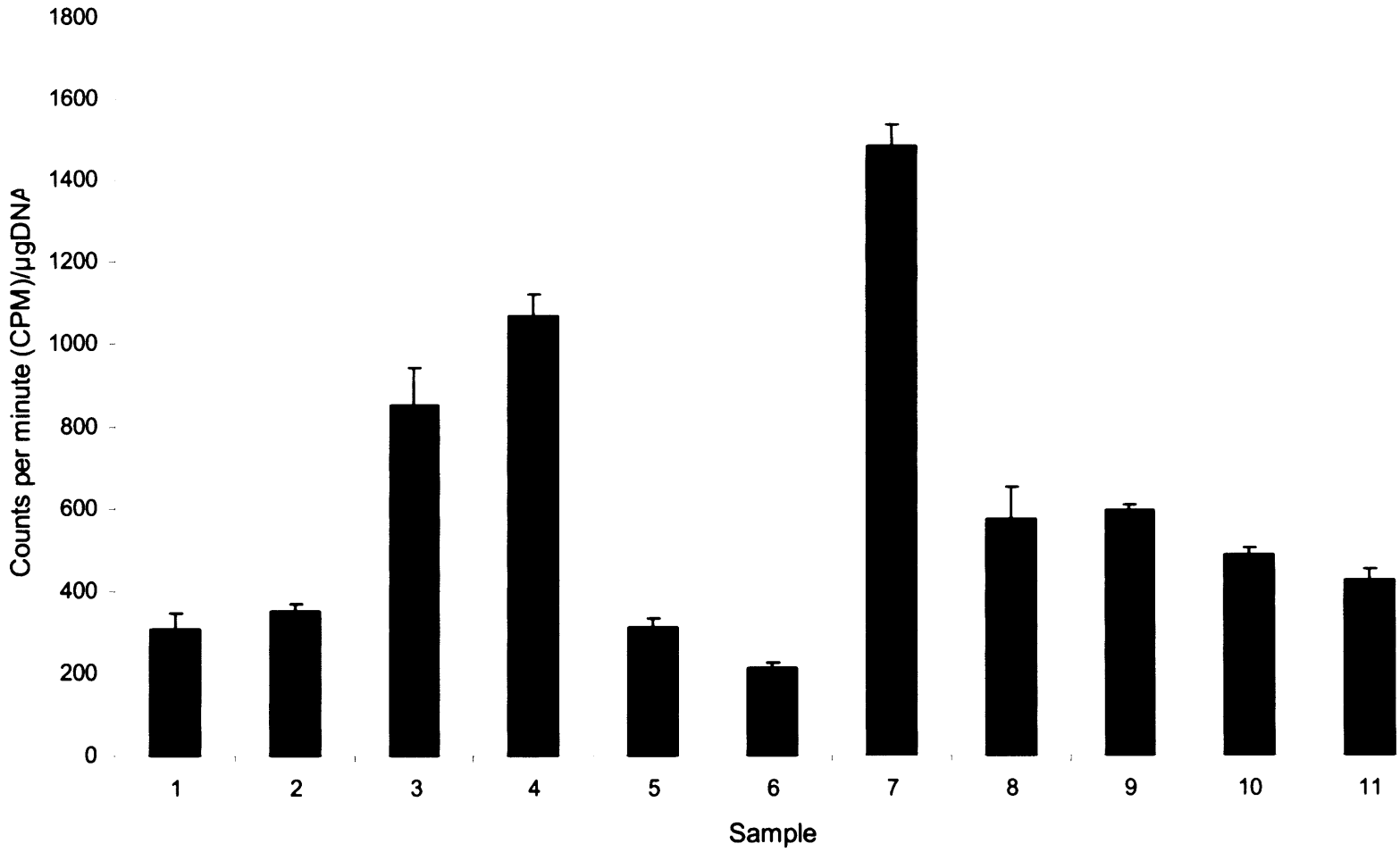



**Figure 5.3** Absolute unloaded controls for $^{35}SO_4$ incorporation in chondrocytes expanded in autologous serum (samples 1-7); fetal calf serum (freshly isolated chondrocytes – sample 8 and passaged chondrocytes - patient 9); and mesenchymal progenitor cells (samples 10 & 11). Each bar represents the mean and standard error of at least 4 replicates.

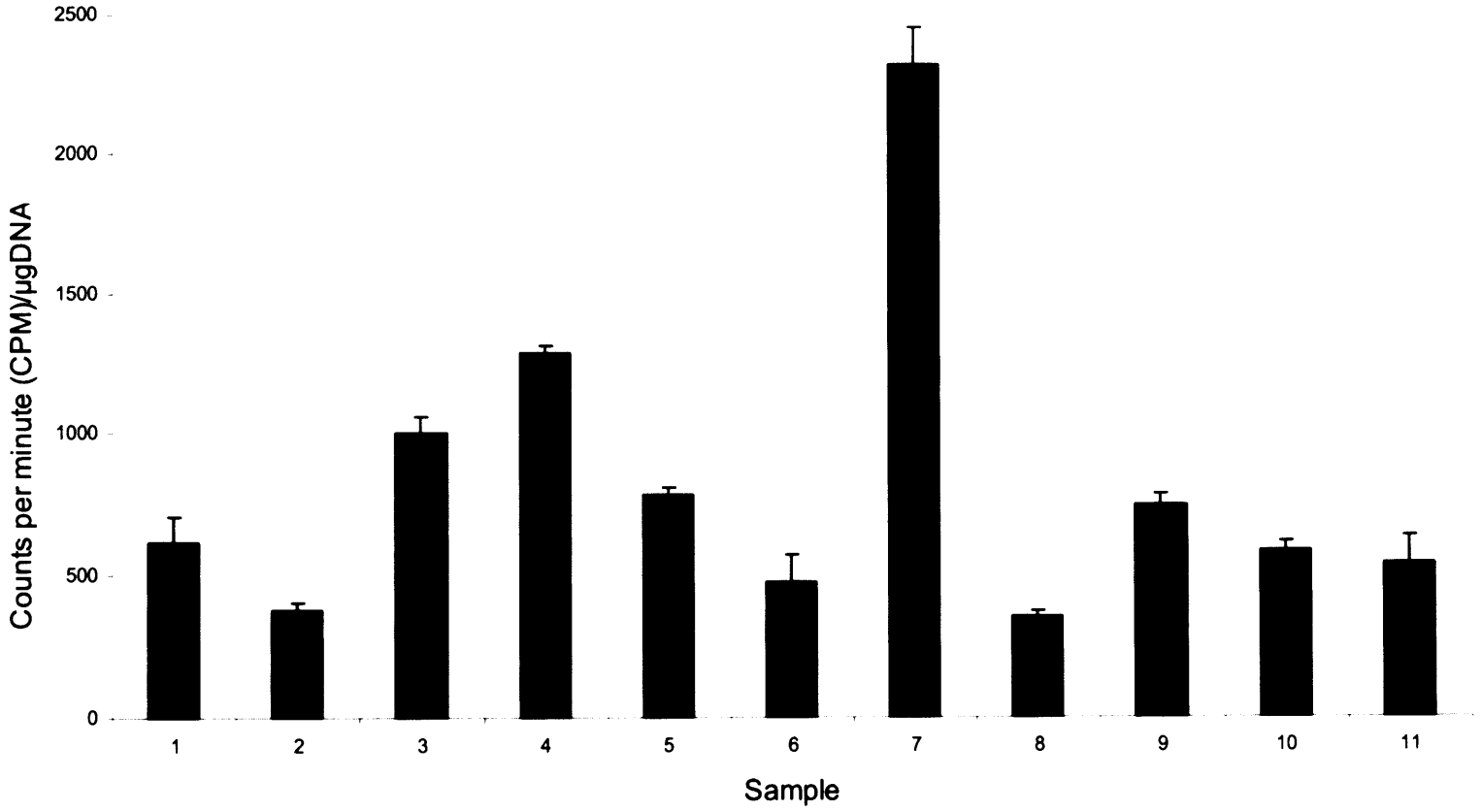



**Figure 5.4** Absolute unloaded controls for [$^3$H]-thymidine in chondrocytes expanded in autologous serum (samples 1-7); fetal calf serum (freshly isolated chondrocytes – sample 8 and passaged chondrocytes - patient 9); and mesenchymal progenitor cells (samples 10 & 11). Each bar represents the mean and standard error of at least 4 replicates.

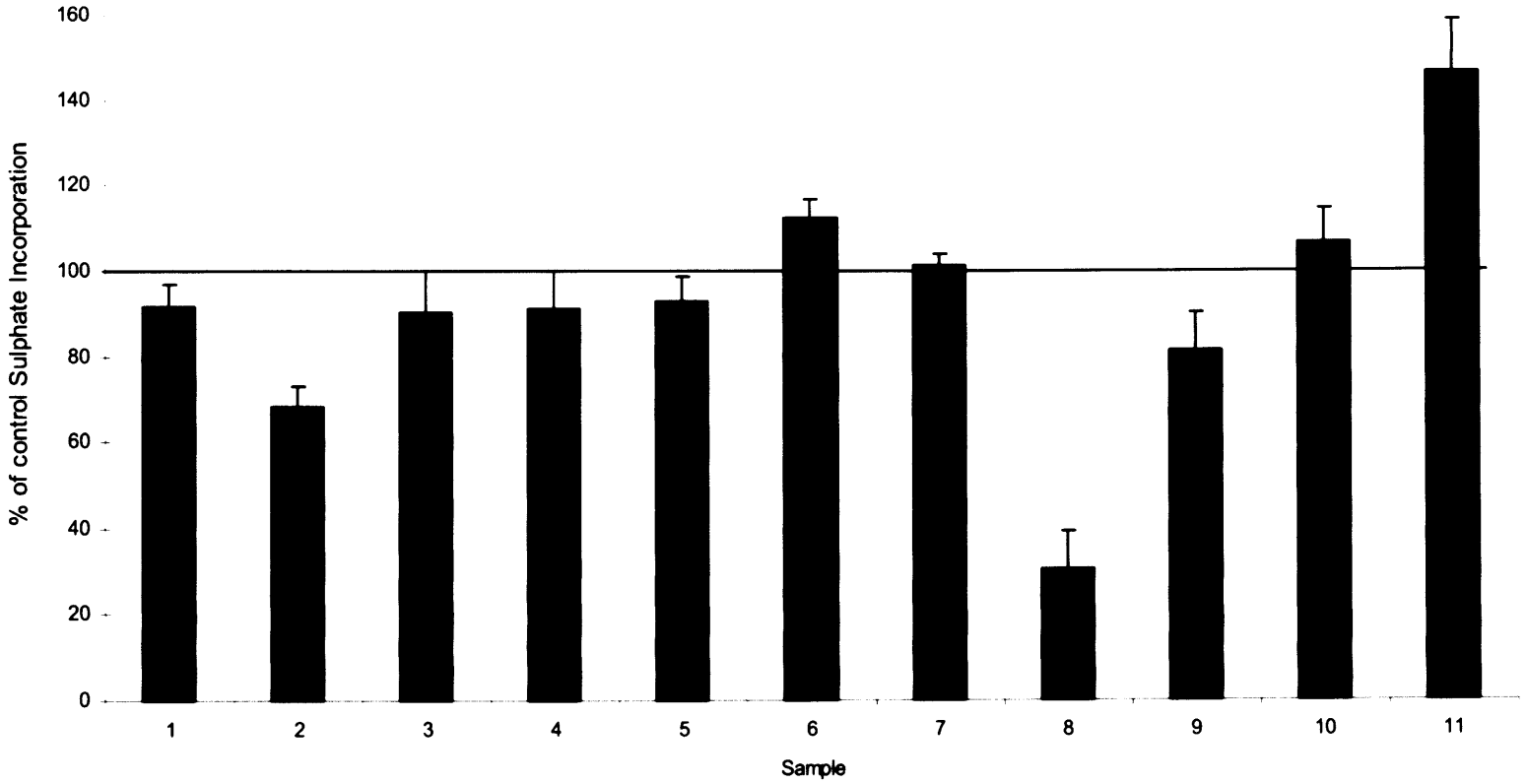



**Figure 5.5** Normalised data for $^{35}SO_4$ incorporation in chondrocytes expanded in autologous serum (samples 1-7); fetal calf serum (freshly isolated chondrocytes – sample 8 and passaged chondrocytes- sample 9); and Mesenchymal Progenitor Cells (samples 10 & 11). Each bar represents the mean and standard error of at least 4 replicates.

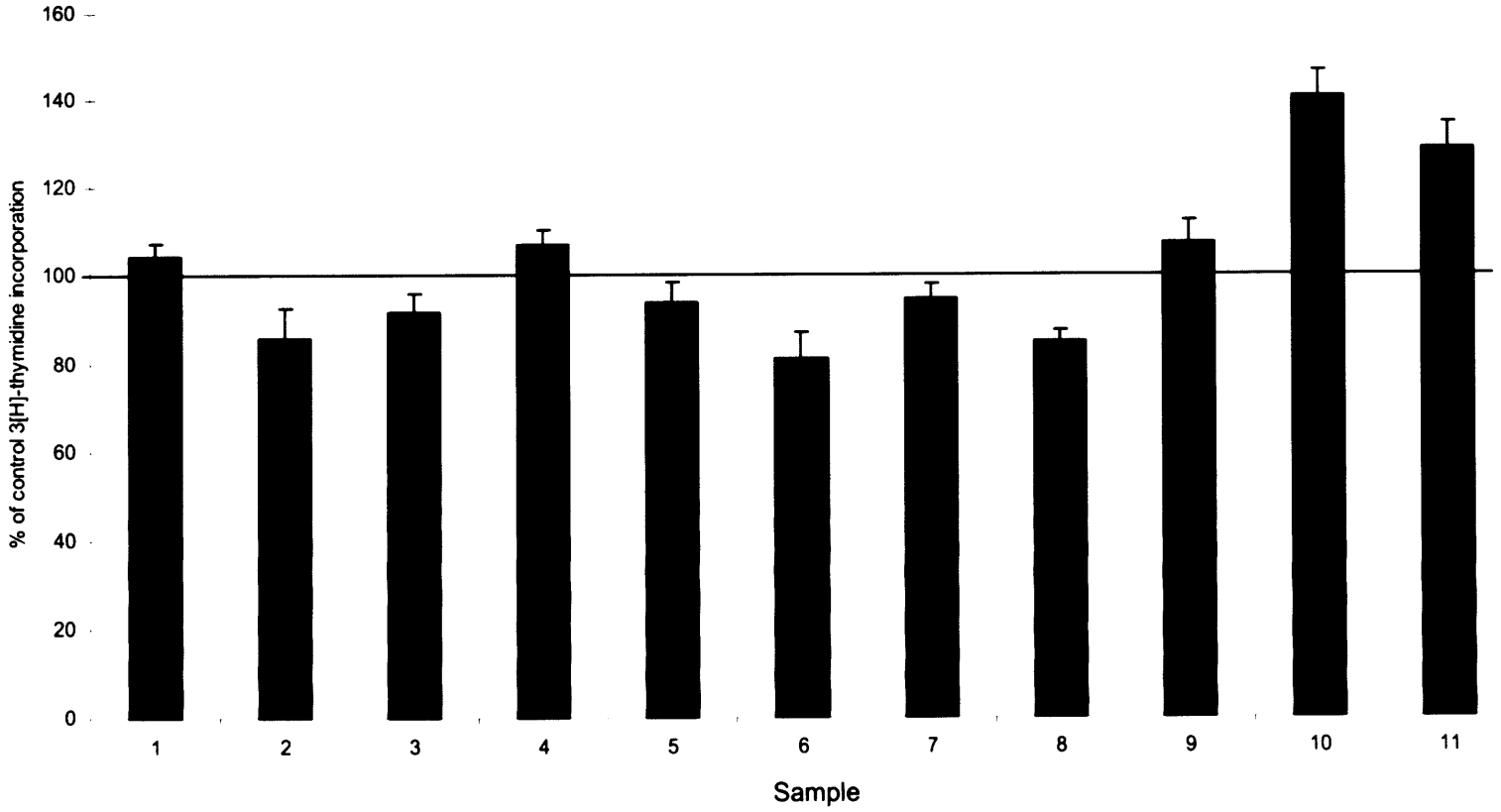



**Figure 5.6** Normalised data for [$^3$H]-thymidine incorporation in chondrocytes expanded in autologous serum (samples 1-7); fetal calf serum (freshly isolated chondrocytes – sample 8 and passaged chondrocytes- sample 9); and Mesenchymal Progenitor Cells (samples 10 & 11). Each bar represents the mean and standard error of at least 4 replicates.

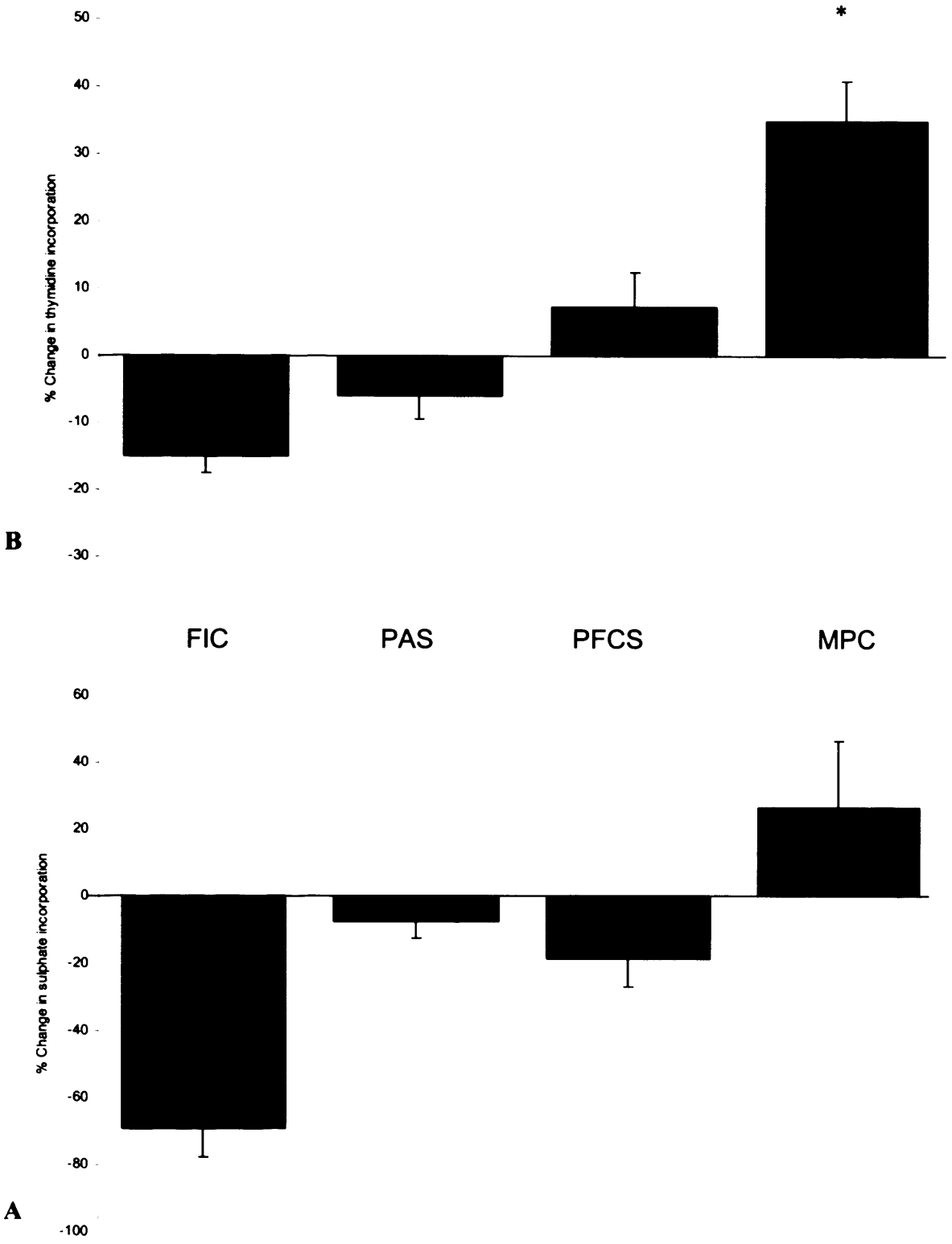



**Figure 5.7** The influence of dynamic compression on $^{35}SO_4$ incorporation (A) and [$^3$H]-thymidine incorporation (B) by freshly isolated chondrocytes (FIC), passaged chondrocytes in autologous serum (PAS), passaged chondrocytes in FCS (PFCS) and mesenchymal progenitor cells (MPC). The cells were embedded in agarose constructs and subjected to 15% dynamic compressive strain amplitude at 1Hz for 48hours. The values are presented as % change from unstrained control levels. Each value represents the mean and standard error of at least 6 replicates. Unpaired Student's *t*-test results indicate no differences from control values unless indicated with an asterix, where $^*p<0.05$).

## 5.4 Discussion

The effects of load on articular cartilage are necessarily complex in nature. A seminal study to visualise and quantify the deformation behaviour of cells *in situ* used light microscopy of a cut cartilage section compressed by up to 30% (Broom and Myers, 1980). This provided the first qualitative evidence that chondrocytes in articular cartilage undergo large changes in shape and intercellular spacing as the extracellular spacing is deformed. Chondrocytes recovered their morphology upon removal of the compression and the orientation of the collagen fibres was also seen to change with compression but recovered on removal of the compression.

Typically, exercise and joint movement leads to an increase in the proteoglycan content, whereas immobilisation leads to a reversible release of proteoglycan (Kiviranta *et al.*, 1988; Palmoski *et al.*, 1979; Saamanen *et al.*, 1990). Experiments *in vitro* have indicated that static loading reduces proteoglycan synthesis, whereas dynamic loading can stimulate synthesis in a frequency-dependant manner (Burton-Wurster *et al.*, 1993; Lee and Bader, 1995b; Lee and Bader, 1997; Lee *et al.*, 1998; Gray *et al.*, 1988; Parkkinen *et al.*, 1993; Sah *et al.*, 1989a; Schneiderman *et al.*, 1986).

The exact mechanisms by which chondrocytes respond to load remain poorly understood. There is evidence that the viscoelastic properties and deformation behaviour of the chondrocyte plays an important role in its interactions with the ECM, and therefore, in the overall regulation of its metabolic activity. It is now believed that chondrocytes undergo significant changes in shape and volume under normal physiological conditions. Presumably, cellular deformation is one of many biophysical factors involved in the regulation of chondrocyte metabolism in response to mechanical stress. The mechanisms of intracellular signaling involved in transducing cellular deformation are not fully understood, but seem to involve

several of the traditional messengers molecules, such as $Ca^{2+}$, $IP_3$, and cAMP as well as less traditional signaling pathways involving the cytoskeleton and nucleus (Guilak, 2000). From theoretical models it is apparent that the mechanical environment of the chondrocyte is strongly influenced by the structure and properties of the chondrocytes, pericellular matrix, and the ECM (Armstrong *et al.*, 1984; Gray *et al.*, 1988; Parkkinen *et al.*, 1993; Schneiderman *et al.*, 1986). In turn, the local osmotic environment within the ECM may influence chondrocyte properties by altering the structure or composition of the chondrocyte cytoskeleton (Guilak, 2000).

In the current study, the effects of dynamic compression on freshly isolated and passaged human chondrocytes together with mesenchymal progenitor cells were assessed. The cells were seeded in agarose constructs cultured in DMEM+10%FCS, and a strain amplitude of 15% at 1Hz was applied. The results are necessarily limited to the availability of cells and so should be regarded as preliminary in nature. Accordingly, only limited inference can be made when comparing the cells from the different sources. The approach used in this experiment has been used previously to reveal the effects of dynamic compression on bovine (Lee and Bader, 1997) and human (Chowdhury, 2002) chondrocytes.

Freshly isolated human chondrocytes obtained from a young male knee (sample 8) revealed a 69% decrease in $^{35}SO_4$ incorporation and a 15% decrease in [$^3$H]-thymidine incorporation between strained and unstrained constructs, although these differences were not statistically significant (see FIC, Figure 5.7). These findings are at variance with those using freshly isolated bovine chondrocytes for which a stimulation of $^{35}SO_4$ incorporation (GAG synthesis) was found at a frequency of 1Hz and a stimulation of [$^3$H]-thymidine incorporation was found at all frequencies of dynamic strain (Lee and Bader, 1995b).

The passaged autologous chondrocytes were cultured using medium supplemented by autologous serum (Verigen Transplantation Service, Leverkeusen, Germany). No information was available as to the exact number of times that each patient's cells had been passaged, although the company stated that generally cells were from P4. No further passages were performed in the host laboratory prior to this experiment. There was a wide variation of absolute $^{35}SO_4$ incorporation data from these seven patients (Figure 5.3). The data were presented in order of ascending age, although no such trends with age could be inferred from the small sample size. Patients 5 and 6 had distinct diagnoses, namely patellofemoral problems. If these data are removed, there does appear to be an apparent trend of increasing $^{35}SO_4$ incorporation with increasing age for the five other patients with suspected femoral condylar problems. This finding must be taken with some caution as it is making an assumption that the underlying clinical diagnosis might have some importance on the behaviour of the patient's cells which is not justified on a scientific basis. For these seven patients, $^{35}SO_4$ incorporation was inhibited in all but one sample (sample 6) in which $^{35}SO_4$ incorporation was stimulated ($p<0.05$). [$^3$H]-thymidine incorporation was inhibited in all but two samples (patients 1 and 4). These findings were not statistically significant ($p>0.05$). In summary the passaged autologous chondrocytes expanded in serum supplemented with autologous serum (samples 1-7) revealed a 7.3% decrease in $^{35}SO_4$ incorporation and a 5% decrease in [$^3$H]-thymidine incorporation between strained and unstrained constructs, although these differences were not statistically significant. These data are summarized in Figure 5.7 (PAS refers to the cells passaged in autologous serum).

The cells taken from the load bearing chondral defect on the medial femoral condyle of patient 9 following monolayer expansion in serum supplemented with fetal calf serum revealed an 18.5% decrease in $^{35}SO_4$ incorporation and a 7.3%

increase in [$^3$H]-thymidine incorporation between strained and unstrained constructs. These differences were not statistically significant (see Figure 5.7. Note PFCS refers to cells passaged in fetal calf serum).

Previous studies have suggested that metabolic activity tends to be relatively low directly following cellular isolation, but increases rapidly after 24-48 hours in culture (Chowdhury, 2002). The modulation in metabolic activity post-isolation is believed to be related to recovery following enzyme treatment, potentially associated with the re-expression of key cell surface markers. Alternatively this process may represent an adaptive response to culture conditions. In a further study, bovine chondrocytes were examined at differing passages and showed a stimulation of [$^3$H]-thymidine and $^{35}SO_4$ incorporation for early passaged (P1-2) chondrocytes but a marked inhibition at P3 and P4 (Wiseman *et al.*, 2004). The authors suggested that the repeated passages and transportation process decrease the activity of the cells and their responsiveness to load. The current study revealed a depression of [$^3$H]-thymidine and $^{35}SO_4$ incorporation for both freshly isolated and passaged chondrocytes, however, differences between the two studies must be noted. In particular, species variation was evident and in the previous study a final cell concentration of $10x10^6$ cells.mL$^{-1}$ in 4% (w/v) agarose type IXa was used, in contrast to the current experiment which used $4x10^6$ cells.mL$^{-1}$ in 3% (w/v) agarose type VII.

Further research investigated the effects of dynamic loading on passaged human autologous chondrocytes of nine patient's cells cultured in a defined media (Chowdhury *et al.*, 2004). In the absence of TGFβ the authors found no significant difference for $^{35}SO_4$ incorporation between strained and unstrained constructs and a marginal increase in [$^3$H]-thymidine incorporation between strained and unstrained constructs of approximately 17% although again this difference was not statistically

significant. Similar findings were observed for the same cells cultured in DMEM+10%FCS (personal communication) which correlates with the findings of this study. The recent study was extended to examine the effects of $10ng.mL^{-1}$ $TGF\beta_3$ on the chondrocytes and revealed a significant stimulation of $[^3H]$-thymidine and $^{35}SO_4$ incorporation over unsupplemented controls. The authors suggested that $TGF\beta_3$ modulates the response of monolayer expanded human chondrocytes to the application of dynamic compression (Chowdhury *et al.*, 2004).

Previous work in the host laboratory also investigated the response of cells from different layers within bovine articular cartilage to mechanical compression (Lee *et al.*, 1998). It was shown that superficial cells (Zone I and some Zone II) exhibited a general inhibition of glycosaminoglycan synthesis on mechanical compression whereas cells from the deeper layers (Zone III and some Zone II) revealed a highly significant 50% stimulation of glycosaminoglycan synthesis on dynamic strains of frequencies of 1Hz. By contrast, proliferation of superficial cells was stimulated by dynamic strain whereas deep cells were not influenced. Therefore, it was suggested that cells in different sub-populations of chondrocytes within the full-depth cell isolate respond differently to mechanical load. In the present study it might be suggested that sample 9 included cells primarily from the deeper layers of articular cartilage (zone II & III). In this study however, these cells responded to loading by stimulation of proliferation ($[^3H]$-thymidine incorporation) but slight depression of proteoglycan synthesis ($^{35}SO_4$ incorporation). These cells had been passaged whereas in the bovine experiments the cells had been freshly isolated (Lee *et al.*, 1998). The tissue from the defect is likely however, to contain a homogeneous collection of cells from all zones and so one possible theory to explain these findings is that the cells are displaying an increased capacity to proliferate in an attempt to heal the defect. Further work is clearly needed in this area.

**Mesenchymal Progenitor Cells**

In the current study, mesenchymal progenitor cells from two patients (samples 10 and 11), subjected to mechanical load in agarose constructs, demonstrated an approximate 27% increase in $^{35}SO_4$ incorporation between strained and unstrained constructs and an increase of 35% in [$^3$H]-thymidine incorporation between strained and unstrained constructs (see Figure 5.7). The thymidine changes were statistically significant ($p<0.05$) but the sulphate changes did not reach statistical significance. The cells were cultured in DMEM+10%FCS in the absence of any defined bioactive factors. (Note that in Figure 5.7, MPC refers to mesenchymal progenitor cells).

Mauck *et al,* using human multipotential mesenchymal cells in alginate disks, showed that mechanical preconditioning, in the presence of a TFG$\beta_1$, enhanced chondrogenic differentiation (Mauck *et al.*, 2003). In an alternative approach, mesenchymal progenitor cells were subjected to cyclic mechanical compression in biodegradable scaffolds (hyaluronan-gelatin composites) in a defined, serum-free chondrogenic medium (Angele *et al.*, 2003). They revealed that loaded aggregates demonstrated significant increases in proteoglycan and collagen contents compared to unloaded controls.

A study assessing the effects of cyclic compressive loading on rabbit bone-marrow mesenchymal stem cells, suggested that compressive loading induces chondrogenic differentiation of rabbit mesenchymal cells (Huang *et al.*, 2004). The authors suggested that cyclic compressive loading promoted chondrogenesis by inducing the synthesis of TGF$\beta_1$, which could then stimulate the cells to differentiate into chondrocytes. In the current study, mesenchymal progenitor cells were cultured

in DMEM+10%FCS in the absence of TGF$\beta_1$ which therefore supports these findings. The previous authors hypothesise that, using the correct mechanical loading regime, undifferentiated cells can be induced to differentiate along the chondrogenic lineage in preparation for *in vivo* implantation. In the present study no attempt was made to analyse the samples for chondrogenic markers such as aggrecan or collagen type II, however, this is an essential next step and forms the basis for further much needed work.

# CHAPTER 6 – FINAL DISCUSSION

*"From Hippocrates to the present age, it is universally allowed that ulcerated cartilage is a troublesome thing and that when once destroyed, it is not repaired"* (Hunter, 1743).

In 1743, twenty five year old William Hunter presented this statement to the Royal Society. Despite going on to become an eminent gynaecologist (Buchanan *et al.*, 1987) his work on cartilage is often accredited to his younger and perhaps more famous brother, John. Nonetheless two and a half centuries later, his conclusions continue to plague Orthopaedic surgeons and researchers alike.

Articular cartilage is avascular, alymphatic and aneural and, as such, has limited intrinsic repair potential (Buckwalter and Mankin, 1998). Surprisingly, however, chondrocytes can be very active metabolically and have a glycolytic rate per cell similar to that of cells in vascularized tissues. However, the total metabolic activity of the tissue is low because of its inherent low cell density (Buckwalter and Mankin, 1997a).

A number of treatments have been proposed, each attempting to provide a long lasting solution to the repair of cartilage damage. Stimulation techniques using electricity (Lippiello *et al.*, 1990) and lasers (Hardie *et al.*, 1989), failed to acquire much credibility. Carbon fibre grafts had some good data published but the reality was that there were so many complications that the technique was abandoned (Brittberg *et al.,* 1994a; Pongor *et al.,* 1992). Techniques used to alter the dynamics of the joint loading, such as osteotomy, offer reasonable medium term results (Insall *et al.*, 1984) but, in the long term, results deteriorate (Rinonapoli *et al.*, 1998).

Techniques involving mesenchymal progenitor cells including perforation of subchondral bone (Insall, 1967; Steadman *et al.*, 1998; Dandy, 1986), and the use of periosteal (Rubak *et al.,* 1982a; Rubak *et al.*, 1982b) or perichondrial grafts (Homminga *et al.,* 1990; Bouwmeester *et al.,* 1997) have always been accepted to result in the formation of fibrocartilage (Insall, 1967; Mankin, 1991; Meachim, Roberts, 1971; Shapiro *et al.*, 1993).

More recent attention has turned to osteochondral allografting, termed mosaicplasty (Hangody and Fules, 2003) and cell transplantation (Brittberg *et al.*, 1994b; Peterson *et al.*, 2000). In the former technique, small-sized multiple cylindrical grafts of bone and its overlying cartilage are transferred from a non articulating area of the knee to the load bearing defect, akin to robbing "Peter to pay Paul". In the latter technique, mature cells are taken from a healthy non-load bearing area of the knee, multiplied *in vitro* and then reimplanted into the load bearing cartilage defect. In Sweden, autologous chondrocyte implantation (ACI) combined with a periosteal graft has so far been used on more than 1200 patients, and, worldwide, variants of autologous chondrocyte transplantation or implantation have been performed in more than 10,000 patients (Brittberg *et al.*, 2003). Using mosaicplasty, good-to-excellent results were reported in 92% of patients at 10 years for femoral condylar lesions (Hangody and Fules, 2003) and ACI yielded good to excellent clinical results in 92% of isolated femoral condyle lesions at a follow up of 2-9 years (Peterson *et al.*, 2000). Nonetheless, good results were published for woven carbon fibers scaffolds, with 83% good or excellent results reported at 2 year follow up (Brittberg *et al.*, 1994a). This technique was, however, abandoned due to poor longer term results and complications, which rarely get published. Similarly, 80% of patients treated with microfracture were reported as rating themselves as "improved" at 7 years (Steadman *et al.*, 2003). The common theme amongst the above results is that they are being reported by the proponents of the technique.

Further data is needed from independent centres to verify the efficacy of the technique and its long term role in the management of articular cartilage injuries.

Indeed, the published data on the outcomes of cartilage repair must be taken with some caution as they are difficult to interpret in the light of good to excellent results in invariably 80% or more of patients in most studies. To date no good prospective randomized studies that compare the treatment group to the natural history of the condition have been reported making the clinical data difficult to interpret. Fifty years ago, Pridie pointed out that there was little correlation between a patient's symptoms and the clinical findings (Insall, 1967). He referred to the gross appearance of what he suggested looked like the perfect repair yet the patient was still debilitated and in need of a knee arthrodesis. Since this time there has been little advancement of our knowledge on the correlation between clinical findings and patient outcome. It has been suggested that a biopsy revealing a hyaline-like repair tissue correlated with the successful clinical results in 80% of biopsies, however, only 37% of patients underwent a biopsy (Peterson, 1998). Indeed the present author witnessed a poor appearance to the repair two years following ACI, with breakdown, fibrillation and fissuring of the tissue and yet the patient was delighted with the surgery and could not have been happier.

In the midst of this uncertainty, clinicians and researchers alike are searching for innovative ways to enhance the current techniques in order to consistently and reproducibly improve the outcomes of cartilage repair.

In the current study when bovine and human chondrocytes were cultured in monolayer, multiplication was rapid although the cells adopted a flattened, fibroblastic, dedifferentiated appearance. Previous studies have revealed that this also leads to a switch in expression from type II to type I collagen (Holtzer *et al.*,

1960; Mayne *et al.*, 1976; Benya *et al.*, 1978; Marlovits *et al.*, 2004). In this study, human chondrocytes that had been monolayer expanded in media supplemented with fetal calf serum were subsequently capable of forming 3D aggregates in a similar medium. In contrast human chondrocytes expanded in monolayer in media supplemented with autologous serum would subsequently not form pellets in media supplemented with fetal calf serum. However, their ability to pellet could be returned by culture for one further passage in media supplemented with fetal calf serum or if the pelleting medium was supplemented by a defined medium containing $TGF\beta_1$. These findings suggest that expression of specific adhesion molecules or surface markers may be altered by the culture conditions used during monolayer expansion and may be involved in the process of aggregation. These findings support two previous studies (Jakob *et al.*, 2001; Chimal-Monroy and Diaz de Leon, 1999), who both suggested that TGF-β isoforms play an important role in the establishment of cell-cell and cell-ECM interactions during precartilage condensations, through differential enhancement of expression of N-cadherin, N-CAM, fibronectin and tenascin.

Once aggregates had formed the bovine chondrocytes redifferentiated in DMEM+10%FCS, however, the human chondrocytes pre cultured in autologous serum, required a defined medium enhanced with $TGF\beta_1$ in order to redifferentiate. The human chondrocyte pellets cultured in DMEM/ITS+/$TGF\beta_1$ were larger with greater amounts of DNA and GAG compared to pellets maintained in DMEM/10%FCS and histologically the pellets maintained in DMEM/ITS+/$TGF\beta_1$ possessed an extensive ECM that stained metchromatically (Figure 3.10) and was positive for type-II collagen. This reaffirms previous findings suggesting that other factors are necessary to enable the redifferentiation process (Benya and Shaffer, 1982). The process appears to involve a combination of microfilament structure modification in association with the effects of bioactive factors, such as $TGF\beta_1$, and

the present findings suggest that the process of aggregation and redifferentiation might be uncoupled.

The present study confirms that a defined medium containing $TGF\beta_1$ induces the re-expression of a chondrocytic phenotype and the subsequent stimulation of GAG and type II collagen production by human monolayer expanded chondrocytes. Accordingly, $TGF\beta_1$ may be necessary for the formation of cellular aggregates, the initiation of cell proliferation and the development of hyaline cartilage matrix within a clinical repair situation. *In vivo*, small amounts of TGFβ could be derived from the periosteum, synovial fluid or the chondrocytes themselves and it is possible that one of the causes of clinical failure is the lack of availability of TGFβ at the site of repair.

In the present study, cells were expanded in monolayer using culture medium supplemented with autologous serum. Although the original description of the ACI technique also used autologous serum (Brittberg *et al.*, 1994b), this practice has not subsequently been standardised in clinical use, where supplementation with either autologous serum or fetal calf serum are employed by different commercial suppliers. The findings in this study suggest that the environment in which the cells are cultured can affect their ability to aggregate and redifferentiate subsequently and so different media could potentially have important clinical consequences.

There are two further concerns pertaining to the site of harvest of the biopsy in ACI. One concern is that the cells are from a non load bearing area of the knee and there is, therefore, a possibility that the cells from this area could behave differently to mechanical load than cells that have been exposed to load *in vivo*. This will be discussed in more detail below. The second concern is the possible morbidity that could result from the biopsy. The process of sacrificing an area of healthy cartilage

in order to repair another part of the knee is an unpleasant one for any surgeon and an alternative source of cells would be attractive.

In this study, it has been shown that the cartilage that is cored out from the defect as part of the second operative procedure contains viable chondrocytes, that following monolayer expansion, were capable of redifferentiating and producing chondrogenic tissue. When cultured as pellets in DMEM/ITS+/TGF$\beta_1$ the cells appeared indistinguishable from those harvested from the usual healthy cartilage from the medial femoral condyle. In this study the cell expansion time for defect derived cells (HDDC) was slow (55 times in 44 days), largely as a result of a long lag time to first passage and might reflect the batch of serum used in culture or a lack of articular cartilage progenitor cells in the repair tissue. Further exploration of this area is warranted.

The second source of cells that was explored in the current study was human mesenchymal progenitor cells. Mesenchymal progenitor cells with chondrogenic potential are present in many tissues of the body. Those of the bone marrow are of particular interest because of the extensive reserve of these cells, their ease of harvest and their expandability in culture. The repair of articular cartilage defects extending beyond the subchondral bone requires mobilization and differentiation of these mesenchymal progenitor cells. The differentiation has to be carefully controlled to give the appropriate repair, with the right mix of chondrogenesis, conversion to hypertrophic chondrocytes and subsequent endochondral ossification below the tidemark. This has never been achieved *in vivo* to date but would theoretically constitute the perfect repair.

In this study an *in vitro* system was used to promote the chondrogenic potential of these cells allowing for study of some of the factors responsible for this complex

process. Human mesenchymal progenitor cells were harvested from the iliac crests of healthy volunteers, isolated by self selection through monolayer culture and induced to chondrogenic differentiation in a pellet model using culture in a chemically defined serum-free medium.

Within two weeks the cells within the pellets displayed a chondrocytic appearance, stained metachromatically with toluidine Blue and were positive for type-II collagen; all features of hyaline cartilage and could perhaps be said to resemble the precartilage condensation of cells in the forming limb bud (Fell, 1925; Newman and Frisch, 1979). The mean GAG content of the pellets increased significantly by 58%, within two weeks in pellet culture, most likely due to an accumulation of extracellular matrix rather than cell proliferation as DNA content did not increase accordingly.
It must be noted, however that only GAG in the pellet was measured and not in the medium. These results might therefore, be confounded if the GAG was being produced but then released into the medium and hence the author recommends that in future study the experiments should be carried out by measuring the total GAG in the system in addition to the GAG in the pellets.

In chapters three and four, the figures discussed were mean values of µg GAG and µgDNA. It might be more appropriate to express these data using a ratio of µgGAG/µgDNA. After 14 days in pellet culture, human autologous chondrocytes obtained from Verigen, and subsequently cultured in DMEM/ITS+/TGF$\beta_1$ revealed 5.2 µgGAG/µgDNA in comparison to the same cells, pelleted in DMEM/10%FCS, which measured 3.6 µgGAG/µgDNA, demonstrating a mean reduction of 1.6 µgGAG/µgDNA when cultured in serum supplemented with bovine serum. In comparison, pellets derived from cells from the defect, expanded (for 3 passages) in DMEM/10%FCS, revealed 4.6 µgGAG/µgDNA. This could be explained by differening batches of culture media (Lennon *et al.*, 1996), and reinforces that the

culture media used to expand the cells could have important implications on the ability of the cells to subsequently re-differentiate and produce GAGs (Goldberg et al, 2005).

In addition the chondrogenic potential of these cells appeared to be retained through multiple passages which is an important attribute if these cells are to be multiplied in numbers and used for repair techniques in clinical practice. The addition of mechanical loading to this model has been shown to stimulate chondrogenesis greater than growth factors alone (Mauck *et al.*, 2003; Angele *et al.*, 2003; Huang *et al.*, 2004). Huang *et al* suggested that cyclic compressive loading promoted chondrogenesis by inducing the synthesis of $TGF\beta_1$, which then could stimulate the cells to differentiate into chondrocytes (Huang *et al.*, 2004).

In the current study, the mesenchymal progenitor cells demonstrated an approximate 27% increase in $^{35}SO_4$ incorporation between strained and unstrained constructs and an increase of 35% in [$^3$H]-thymidine incorporation between strained and unstrained constructs. The thymidine changes were statistically significant ($p<0.05$) but the sulphate changes did not reach statistical significance. Note that the mesenchymal progenitor cells in this study were compressed in agarose in the presence of DMEM+10%FCS and not in a growth factor enhanced defined medium. This is an important finding as it reinforces the theory that mechanical load could influence the differentiation of stem cells along a particular lineage. *In vivo* studies have suggested that mechanical factors are involved in the regulation of the morphology and biochemical composition of tendons that wrap around bones (Giori *et al.*, 1993) and in a similar manner tissues with the ideal biomechanical composition for articular cartilage could be created if it were possible to identify the appropriate mechanical and biological factors necessary.

The effect of mechanical loading on cartilage repair has received insufficient attention to date. The rehabilitation of patients who have undergone ACI is clearly very important and yet the literature is conflicted even on this issue. Brittberg *et al* advocate the use of continuous passive motion on the first postoperative day and continues while the patient stays in the hospital for two or three days. In contrast Bentley *et al* (Bentley *et al.*, 2003) and Haddo *et al* (Haddo *et al.*, 2004) placed their patients in a plaster cast to prevent any flexion or extension for the first two postoperative weeks. The justification for this is to prevent graft disturbance. Both investigators agree that weight bearing is beneficial however, and should be started early. Unfortunately, protocol design issues like these make interpretation and comparison of studies in this area difficult.

In the current study the effects of dynamic compression on freshly isolated and passaged human chondrocytes was also studied. The cells were seeded in agarose constructs and cultured in DMEM+10%FCS under strain amplitude of 15% at 1Hz. Freshly isolated human chondrocytes revealed a 69% decrease in $^{35}SO_4$ incorporation and a 15% decrease in [$^3$H]-thymidine incorporation between strained and unstrained constructs, although these differences were not statistically significant. Chowdhury suggested that the metabolic activity of these cells is likely to be relatively low directly following cellular isolation, but should increase after 24-48 hours in culture due to re-expression of key cell surface markers following the enzyme treatment or as a result of an adaptive response to culture conditions (Chowdhury, 2002).

The passaged autologous chondrocytes in this study (samples 1-7) revealed a 7.3% decrease in $^{35}SO_4$ incorporation and a 5% decrease in [$^3$H]-thymidine incorporation between strained and unstrained constructs, although these differences were not statistically significant (Figure 5.7). When similar human cells

were expanded in monolayer culture in serum supplemented with fetal calf serum however, they revealed an 18.5% decrease in $^{35}SO_4$ incorporation and a 7.3% increase in [$^3$H]-thymidine incorporation between strained and unstrained constructs. These differences were not statistically significant (Figure 5.7).

Wiseman *et al* showed that repeated passages and transportation process decrease the activity of the cells and their responsiveness to load (Wiseman *et al.*, 2004). Chowdhury reported similar finding on passaged human autologous chondrocytes using a medium of DMEM+10%FCS (personal communication). Chowdhury *et al* went on to study the effects of 10ng.mL$^{-1}$ TGF$\beta_3$ on these chondrocytes and revealed a significant stimulation of [$^3$H]-thymidine and $^{35}SO_4$ incorporation over un-supplemented controls. There is obviously a concern that repeated passage of the human chondrocytes might lead to implantation of cells that are less responsive to load. The author also raises the question as to whether the cells that are being used in clinical practice are less likely to redifferentiate in the absence of further mechanical or humoral stimulation. Certainly the findings in this study would suggest that TGFβ is an important factor that might modulate the response of monolayer expanded human chondrocytes both to re-expression of chondrogenic phenotype and to the application of dynamic compression (Chowdhury *et al.*, 2004).

The findings in this study support the work of Yoo *et al* (Yoo *et al.*, 1998) and Mackay *et al* (Mackay *et al.*, 1998) and identify a technique for harvesting bone marrow that is quick and easy to carry out and in the authors' hands without complications. If the technique was ever to be used in clinical practice, it would replace the need for a healthy cartilage biopsy in the first stage of the cartilage replacement procedure. In this situation, the patient would have the bone marrow aspiration performed, possibly under local anaesthetic, four weeks prior to the

definitive operative procedure. The tissue appearance from the *in vitro* model appears to be hyaline cartilage in that it contains abundant matrix staining metachromatically and positive for type II collagens. One fundamental question remains, however, namely why *in vivo* any repair involving mesenchymal progenitor cells has led to the formation of fibrocartilage as opposed to hyaline cartilage.

At the molecular level, a variety of proteins (bioactive factors) must be made and sequentially presented to a large number of specifically positioned undifferentiated progenitor cells. Each molecule acts as only a single note in a symphony of signals that effect the appropriate responding cells to stimulate them to form embryo-like tissue. In addition mechanical forces will assist this process either through mechanotransduction pathways or suggested by Huang *et al* through the induction of synthesis of $TGF\beta_1$, (Huang *et al.,* 2004).

It is postulated that one reason that *in vivo* repairs have always been fibrocartilaginous is that the tissue is not exposed to sufficient mechanical load resulting in a repair that invariably contains a mixture of hyaline and fibrocartilagionus tissue. Figure 4.1 shows that biopsies of the tissue within the symptomatic cartilage defect invariably have a mixed cartilaginous appearance with what appears to be hyaline cartilage at the base and often a fibrocartilaginous cap. The author hypothesises that this could result from a lack of mechanical stimuli in the base of the repair, where loads are shared by the borders of the defect but not by the defect tissue itself. A schematic of this proposal is illustrated in Figure 6.1. As the defect fills with tissue it eventually becomes exposed to mechanical loads and hence begins to form hyaline cartilage. The cells at the joint surface at this time have differentiated and the tissue is already fibrocartilaginous. If the proposal in Figure 6.1 is valid then pre-conditioning repair tissue, such as a collagen mesh embedded with mesenchymal progenitor cells, *in vitro*, could lead to the

development of hyaline cartilage which, when exposed to load *in vivo,* would continue to mature. It is of course also possible that the tissue formed in the defect is fibrocartilaginous and that there is an element of trans-differentiation of fibrocartilage to hyaline cartilage. Further study is recommended.

There is an urgent need for a solution to the problem of cartilage damage. The tools at the disposal of the Orthopaedic surgeon have to date relied on either inadequate attempts at restoration or replacement, neither of which has resulted in the perfect solution. In addition there are no randomized prospective studies that compare the natural history of a repair tissue to that of other forms of repair tissue and therefore, long-term functional outcome remains an uncertainty. Researchers rely on the identification of constituents of normal articular cartilage, such as proteoglycans and collagen type II and this in itself poses a potential pitfall. One could look at the ingredients needed to make a cake, namely eggs, yeast, sugar and water. However, just because you have all of these ingredients does not mean you have a beautiful cake. Likewise just because you can identify collagen type II or aggrecan does not mean you have normal articular cartilage. Brittberg and others have described repair tissue as "hyaline like", which is far from normal articular cartilage (Brittberg *et al.,* 1994a). Sadly this latter point has to date only been demonstrated through the passage of time, with the appearance of softening, breakdown and fibrillation of the repair tissue. The properties of articular cartilage are far greater than the sum of the individual constituents from which it is made and hence it is not only the correct constituents that is needed, but moreso the correct composition, the perfect integration with the underlying subchondral bone and adjacent cartilage and the correct mechanical and biochemical properties.

The bone marrow, subchondral bone, synovium and synovial fluid are also likely to be the other 'secret' ingredients that could assist through the production & release

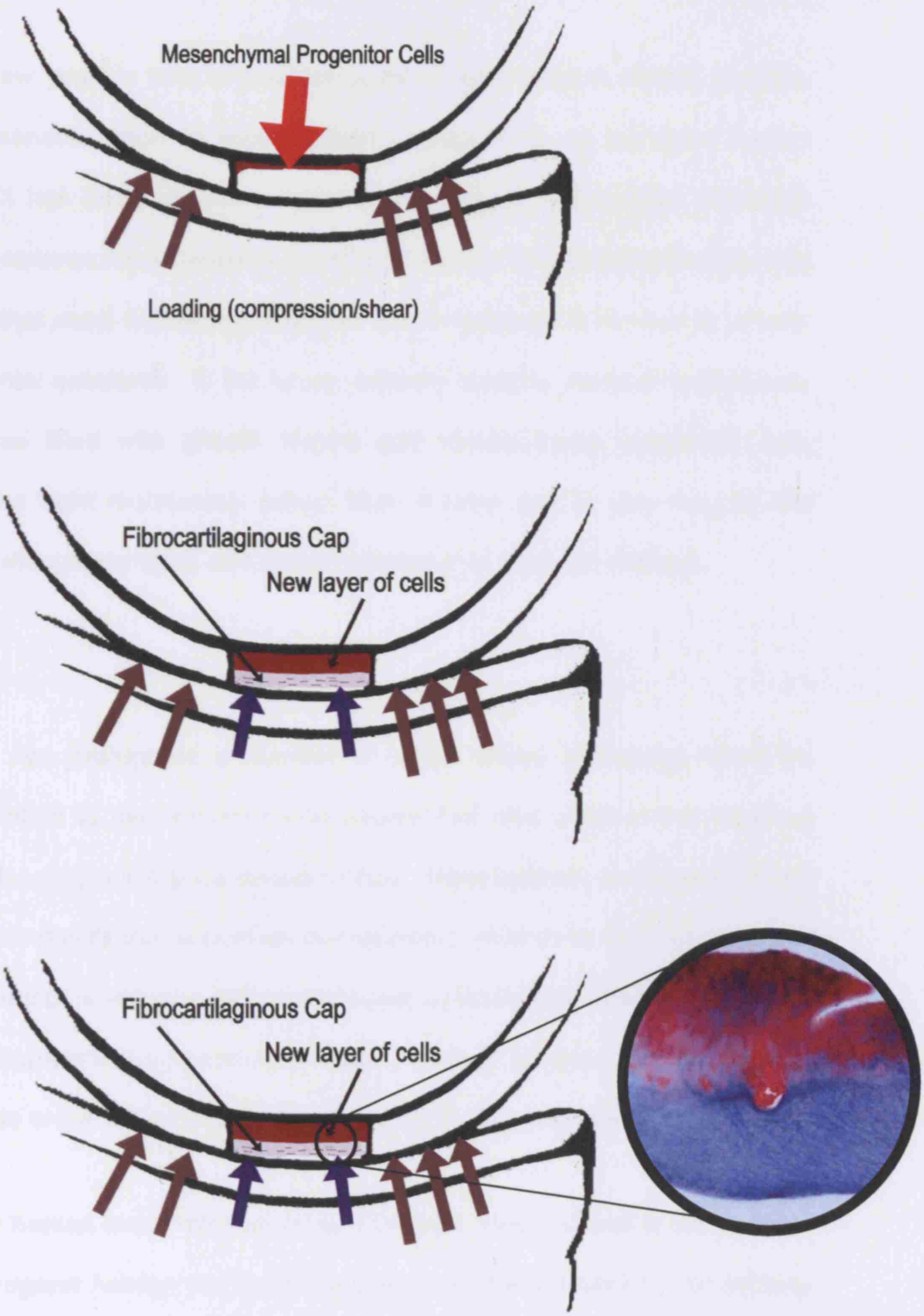



**Figure 6.1** Theory for why a fibrocartilage repair always tends to follow the presence of marrow stimulation technique. Follow the initial damage mesenchymal progenitor cells arrive at the defect with marrow blood (A). These cells are not exposed to any mechanical stimulus which is all taken by the surrounding edge of the defect. As the defect fills and the repair tissue begins to be exposed to mechanical load (B), the deeper tissue is capable of differentiating into hyaline cartilage, whilst the cap of the tissue is already differentiated and remains fibrocartilaginous (C). Inset in the circle demonstrates a typical hyaline/fibrocartilaginous repair seen in a patients defect.

of growth factors such as TGFβ.

Over the last few years a host of treatments have been used in clinical practice, most with reasonable short to medium term results. One of the most current treatments, ACI has been used on over 10,000 patients, yet despite such high volumes of procedures there remain a significant number of unresolved issues with this technique that need further investigation. Much research is needed to answer some fundamental questions. In the future, delivery systems made of synthetically derived matrices filled with growth factors and mesenchymal progenitor cells coupled with the right mechanical stimuli both *in vitro* and *in vivo* may be the solution this challenging enigma and a true restoration of articular cartilage.

**Further work:**

1) This thesis has highlighted a number of areas where knowledge could be improved in relation to the biomechanical events that take place in the repair of cartilage defects using autologous chondrocytes. These include clarification on the optimum number of cells that should be re-implanted; whether to use periosteum or an artificial covering, whether or not sutures have a detrimental effect on the repair and the most appropriate postoperative weight bearing protocol. Further work to investigate these areas will add support to the case for this technique.

2) In this study human mesenchymal progenitor cells were induced to differentiate along a chondrogenic lineage using chemical and mechanical stimuli. An exciting prospect would be to identify the ideal mechanical loading protocol that would be necessary to reliably induce mesenchymal progenitor cells toward a chondrogenic lineage. This would require extensive experimentation using agarose, alginate and possibly scaffold models. The *in vivo* environment may provide many of the signals required for the appropriate differentiation of these cells, but, in the first instance *in*

*vitro* work will be preferable as it enables more systematic investigation of controlled signals.

3) It has been identified that cells from the defect can be a source of autologous cells for the technique of ACI. This study only used one patient and hence this work should be repeated with a larger number of patients to identify the range of times needed for the cellular expansion process.

4) The present study confirms that a defined medium containing TGFβ1 induces a chondrocytic phenotype in de-differentiated human monolayer expanded chondrocytes. Accordingly, TGFβ may be necessary within a clinical repair situation. Further work to identify the optimal isoform of TGF, the appropriate concentration and the best delivery model is needed and will lead to a clearer solution to the challenge of articular cartilage damage.

5) The theory hypothesized in this thesis suggests that a lack of mechanical stimulus could be responsible for the differentiation of marrow progenitor cells into fibrocartilaginous tissue. An exciting project would explore this theory in further depth to ascertain specific stimuli to induce differentiation using specific 3D models of chondrogenesis.

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# Appendix A – PREPARATION OF MATERIALS

**Growth medium preparation (referred to as DMEM/10% FCS)**

440mL Dulbecco's Modified Eagle Medium (DMEM – 31966-021/Life Technology, Paisley, UK)

50mL Fetal Calf Serum (10%(vol/vol)) (FCS - Life Technology, Paisley, UK)

5 mL Penicillin / Streptomycin (50units.mL$^{-1}$ - Life Technology, Paisley, UK)

5 mL MEM

The FCS, antibiotics and MEM were mixed together and filtered through a 0.22 micrometer filter. This filtrate was then added to the DMEM. The final mixture was divided into 60mL aliquots in universal containers and stored at -20°C.

**Defined* Medium**

200mL DMEM without phenol red (DMEM – 11880-028/Life Technology, Paisley, UK)

2mL ITS+ Premix (Becton Dickson Labware, MA, USA)

2mL Sodium Pyruvate()

2mL Penecillin / Streptomycin (50U.mL$^{-1}$ )

1 vial Ascorbate Phosphate 50ηg.mL$^{-1}$ (omitted for experiments with human autologous chondrocytes)

7.85μg Dexamethasone ($10^{-7}$ M)

2μg TGFβ1 (10ηg.mL$^{-1}$ )

The TGFβ was reconstituted in 4mM HCl with 1mg.mL$^{-1}$ bovine serum albumin (BSA). The DMEM for this media is clear and contains: sodium pyruvate, 1000mg/L

glucose and pyridoxine. The ascorbate was not added for the experiments involving human autologous chondrocytes.

**Pronase* preparation**

1g pronase powder (Merck, Poole,UK)

100mL DMEM + 20% FCS

The activity of the pronase was 700U.mL$^{-1}$

**Collagenase* preparation**

0.0781g (100u.mL$^{-1}$) Collagenase Sigma Type XI (Sigma,Poole, UK)

1000mL DMEM + 20% FCS

The activity of the collagenase was 100U.mL$^{-1}$

**Trypsin* preparation**

0.25g Trypsin (Sigma, Poole,UK)

1mL HEPES 1M

100mL PBS (Life Technology, Paisley, UK)

*For each of the above preparations, the constituents were mixed and then filtered through a 0.22μm filter. Aliquots of 20 mL were stored at -20°C.

**Papain Digest Buffer preparation**

| | |
|---|---|
| 1.618g (55mM) | Sodium citrate (Merck, Poole, UK) |
| 0.877g (150mM) | Sodium chloride (Merck, Poole, UK) |
| 0.079g (5mM) | Cysteine Hydrochloride (Merck, Poole, UK) |
| 0.186g (5mM) | EDTA (Merck, Poole, UK) |

0.56 units.$mL^{-1}$ Papain (stored at 4°C) (=1μg.$mL^{-1}$)

The papain digest buffer was prepared by adding these ingredients to 100 mL distilled water. The solution was stored at room temperature. Immediately prior to use papain was added to the buffer, the solution mixed and then filtered through a 0.22μm filter.

**Sodium Citrate Buffer (SSC)**

0.877g (150mM) Sodium chloride

1.47g (50mM) Sodium citrate

The salts were dissolved in 100mL distilled water at pH 7.0. The solution was autoclaved and stored in 50mL glass bottles for use.

**Trypan Blue preparation**

0.4g Trypan Blue

100mL normal saline

This gave a 0.4% solution that was filtered and stored in aliquots of 20mL at room temperature.

**1,9-dimethylene blue dye (DMB)**

0.016g DMB (Sigma, Poole,UK)

5mL Ethanol (Merck, Poole,UK)

2.0g Sodium formate (Merck, Poole,UK)

2mL Formic acid (Merck, Poole,UK)

These ingredients are then made up to 1000mL in distilled water. The pH of the final solution was varied between pH 3.0 – 1.5 by the addition of formic acid drops whilst the solution was being stirred in a pH meter.

**Agarose preparation**

0.32g Type IX-A agarose – Ultra-low gelling temperature (Sigma, UK)

4mL EBSS

The alginate is added to the EBSS and the solution is mixed on a rolamixer for 2 hours. The mixture is then autoclaved and stored at 37°C prior to use. This makes a solution of 8% which will give a final solution of 4% when added to the cell solution.

**Digestion of the agarose plugs**

1mL Papain Digest Buffer (PDB)

10μL Agarase ($10u.mL^{-1}$)

The PDB is added to the agarose plug and placed into a 70°C oven for 1 hour. The mixture is then cooled at 37°C for 10 minutes before the agarase and papain are added. The mixture is then stored at 37°C overnight and the final digestion occurs when placed in a 60°C oven for 1 hour.

**4% Paraformaldehyde in 0.1M cacodylate buffer**

16g Paraformaldehyde dissolved in 200mL distilled water (heated to 60°C whilst stirring)

The solution is then cleared using 1M NaOH.

This 200mL is then made up to 400mL with 0.2M sodium cacodylate buffer at pH 7.4

**DAB Solution preparation**

5 mg 3'3-Diaminobenzidene tetrachloride (DAB)

100μL 1% Hydrogen peroxide (870μL distilled water + 30μL hydrogen peroxide)

10mL Distilled water

Using gloved hands 1 tablet of DAB is added to 10mL distilled water. This solution is then filtered and the 100μL of 0.1% solution of hydrogen peroxide is added just prior to use.

**1,9-DimethylMethylene Blue (DMB) Solution preparation**

| | |
|---|---|
| 0.016g | DMB |
| 5.0mL | Ethanol |
| 2.0g | Sodium formate |
| 2.0mL | Formic acid |

Add together and make up to 990mL with distilled water. Adjust the pH to 3.0 using formic acid. Make final volume up to 1000mL. The solution is then stored in a foil covered bottle at room temperature and immediately prior to use the pH is adjusted by the addition of drops of formic acid to the desired pH.

**Tris buffered saline (TBS) preparation at pH 7.6**

| | |
|---|---|
| 40g | Sodium chloride |
| 3.025g | Tris (trishydroxymethylamine) |
| 22mL | 1M HCl |
| 5000mL | Distilled water |

The mixture was stirred on a pH meter and drops of 1M NaOH was added to get to pH 7.6

**0.1% Trypsin preparation at pH 7.8**

| | |
|---|---|
| 0.1g | Trypsin (Sigma, Poole,UK) |
| 0.1g | Calcium Chloride |
| 100mL | Distilled water |

# Appendix B – ETHICS COMMITTEE APPROVAL

UNIVERSITY COLLEGE LONDON MEDICAL SCHOOL

INSTITUTE OF ORTHOPAEDICS

DIVISION OF ORTHOPAEDICS & LABORATORY SCIENCE

Royal National Orthopaedic Hospital Trust
Brockley Hill
Stanmore
Middlesex HA7 4LP
United Kingdom

Telephone +44 (0)181 954 2300 Ext
Fax +44 (0)181 954 8560
Our Ref: DL/CP

10 July 1998

Professor G Bentley
The Institute of Orthopaedics
RNOH
Stanmore

Dear Professor Bentley

I am delighted to inform you that your project entitled *'Prospective Clinical Trial of Articular Cartilage Repair Methods'* has been approved by the Joint Research and Ethical Committee subject to the following changes being made.

Yours sincerely

**DR DAVID LEE**
**Secretary of**
**The Joint Research and Ethical Committee**

Enc.

Royal National Orthopaedic Hospital NHS
NHS Trust

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Tel: 020 8954 2300
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07 March 2005

Dr Andy Goldberg
Specialist Registrar
Joint Reconstruction Unit
RNOH

Dear Dr Goldberg

**Re: Use of human autologous mesenchymal progenitor cells in cartilage repair techniques**

Thank you for providing the information about your research project, which involved taking bone marrow biopsies from patients (who had consented to be in a research study), and using these samples to obtain mesenchymal stem cells, that were then cultured to define best methods of growing these cells.

Unfortunately, our records from that period are incomplete, and I am unable to provide you with the original approval letter. However, the study conformed to standard practice at the time (patients were asked to consent for additional tissue to be taken and their tissue samples were anonymised).

I am therefore happy that the project would have been approved when it came before the Joint Research Ethics Committee.

I will of course inform the Committee of my decision.

Yours sincerely

**Dr J Berman**
**Chairman, JREC 1999-2003**

# Appendix C – ICRS EVALUATION SCORING SYSTEM

# ICRS Cartilage Injury Evaluation Package

Consists of two parts:

**A: PATIENT PART:**
ICRS Injury questionnaire
The IKDC Subjective Knee Evaluation Form-2000

**B: SURGEONS PART**
ICRS Knee Surgery History Registration
IKDC KneeExamination form-2000
ICRS- Articular cartilage injury mapping system
ICRS-Articular cartilage injury classification
ICRS-Osteochondritis dissecans classification
ICRS-Cartilage Repair Assessment system

The ICRS Clinical Cartilage Injury Evaluation system-2000 was developed during ICRS 2000 Standards Workshop at Schloss Münchenwiler, Switzerland, January 27-30, 2000 and further discussed during the 3rd ICRS Meeting in Göteborg, Sweden, Friday April 28, 2000.
The participants in the Clinical Münchenwiler Evaluation Group were as follows:

Chairman Mats Brittberg, Sweden
Paolo Aglietti, Italy
Ralph Gambardella, USA
Laszlo Hangody, Hungary
Hans Jörg Hauselmann, Switzerland
Roland P Jakob, Switzerland
David Levine, USA
Stefan Lohmander, Sweden
Bert R Mandelbaum, USA
Lars Peterson, Sweden
Hans-Ulrich Staubli, Switzerland

There was a discussion regarding the use of IKDC-1999 vs KOOS (**K**nee Injury and **O**steoarthritis **O**utcome **S**core). The decision in Göteborg was to continue with IKDC (IKDC representatives: A. Anderson, R. Jakob, H.-U. Stäubli) but there will also be comparative studies with the KOOS (http://www.koos.nu/)

The clinical evaluation system can also be combined with the ICRS Imaging Protocol as well as the ICRS Biomechanical Protocol

Comments on the ICRS Cartilage Evaluation forms to:

# ICRS – CARTILAGE INJURY STANDARD EVALUATION FORM-2000
## PATIENTS PART

**Patient Name:**_____

**Birthdate : Day**_____**Month**_____**Year**_____

Street:_____ _____ Zip:_____Town:_____Country:_____

Phone:_____E -mail:_____

Gender:_____

Height:_____cm Weight:_____Kg

Examiner:_____Date of examination:_____

**Localisation:**

Involved knee: Right ___ Left___

Opposite knee: Normal__ Nearly Normal__Abnormal__Severely abnormal__

**Onset of symptoms**

(date):_____ Gradual:_____Acute:_____

Etiology/Cause of injury:

**Activity at injury:**

Activity of daily living:_____Sports_____

Traffic_____Type of vehicle_____ Work_____

| **Activity-level:** | **before injury** | **Just now prior to surgery** |
|---|---|---|
| I: high competitive sportsman/woman | yes___No___ | yes___No___ |
| II: well-trained and frequently sporting: | yes___No___ | yes___No___ |
| III: sporting sometimes | yes___No___ | yes___No___ |
| IV: Non-sporting | yes___No___ | yes___No___ |

**Functional status**

I: I can do everything that I want to do with my joint
II: I can do nearly everything that I want to do with my joint
III: I am restricted and a lot of things that I want to do with my joint are not possible
IV: I am very restricted and I can do almost nothing with my joint without severe pain and disability

| | |
|---|---|
| Preinjury: | I___II___III___IV___ |
| Just prior to surgery | I___II___III___IV___ |
| Present activity level | I___II___III___IV___ |

# IKDC CURRENT HEALTH ASSESSMENT FORM *
## Patients Part:

**Your Full Name** _____

**Your Date of Birth** _____/_____/_____
Day Month Year

**Today's Date** _____/_____/_____
Day Month Year

**1. In general, would you say your health is:**

- ❑ Excellent
- ❑ Very Good
- ❑ Good
- ❑ Fair
- ❑ Poor

**2. Compared to one year ago, how would you rate your health in general now?**

- ❑ Much better now than 1 year ago
- ❑ Somewhat better now than 1 year ago
- ❑ About the same as 1 year ago
- ❑ Somewhat worse now than 1 year ago
- ❑ Much worse now than 1 year ago

**3. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?**

| | | Yes, Limited A Lot | Yes, Limited A Little | No, Not Limited At All |
|---|---|---|---|---|
| a. | Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports | ❑ | ❑ | ❑ |
| b. | Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf | ❑ | ❑ | ❑ |
| c. | Lifting or carrying groceries | ❑ | ❑ | ❑ |
| d. | Climbing several flights of stairs | ❑ | ❑ | ❑ |
| e. | Climbing one flight of stairs | ❑ | ❑ | ❑ |
| f. | Bending, kneeling or stooping | ❑ | ❑ | ❑ |
| g. | Walking more than a mile | ❑ | ❑ | ❑ |
| h. | Walking several blocks | ❑ | ❑ | ❑ |
| i. | Walking one block | ❑ | ❑ | ❑ |
| j. | Bathing or dressing yourself | ❑ | ❑ | ❑ |

4. **During the <u>past 4 weeks</u>, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?**

| | | YES | NO |
|---|---|---|---|
| a. | Cut down on the amount of time you spent on work or other activities | ❑ | ❑ |
| b. | Accomplished less than you would like | ❑ | ❑ |
| c. | Were limited in the kind of work or other activities | ❑ | ❑ |
| d. | Had difficulty performing the work or other activities (for example, it took extra effort) | ❑ | ❑ |

5. **During the <u>past 4 weeks</u>, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?**

| | | YES | NO |
|---|---|---|---|
| a. | Cut down on the amount of time you spent on work or other activities | ❑ | ❑ |
| b. | Accomplished less than you would like | ❑ | ❑ |
| c. | Didn't do work or other activities as carefully as usual | ❑ | ❑ |

6. **During the <u>past 4 weeks</u>, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?**

❑Not At All
❑Slightly
❑Moderately
❑Quite a Bit
❑Extremely

7. **How much bodily pain have you had during the <u>past 4 weeks</u>?**

❑None
❑Very Mild
❑Mild
❑Moderate
❑Severe
❑Very Severe

8. **During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?**

❑Not at All
❑A Little Bit
❑Moderately
❑Quite a Bit
❑Extremely

9. **These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the <u>past 4 weeks</u>...**

| | All of the time | Most of the time | A good bit of the time | Some of the time | A little of the time | None of the time |
|---|---|---|---|---|---|---|
| a. Did you feel full of pep? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| b. Have you been very nervous? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| c. Have you felt calm and peaceful? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| d. Did you have a lot of energy? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| e. Have you felt down-hearted and blue? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| f. Did you feel worn out? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| g. Have you been a happy person | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| h. Did you feel tired? | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |

10. **During the <u>past 4 weeks</u>, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?**

❑All of the time
❑Most of the time
❑Some of the time
❑A little of the time
❑None of the time

11. **How TRUE or FALSE is each of the following statements for you?**

| | Definitely True | Mostly True | Don't Know | Mostly False | Definitely False |
|---|---|---|---|---|---|
| a. I seem to get sick a little easier than other people | ❑ | ❑ | ❑ | ❑ | ❑ |
| b. I am as healthy as anybody I know | ❑ | ❑ | ❑ | ❑ | ❑ |
| c. I expect my health to get worse | ❑ | ❑ | ❑ | ❑ | ❑ |
| d. My health is excellent | ❑ | ❑ | ❑ | ❑ | ❑ |

# 2000 IKDC SUBJECTIVE KNEE EVALUATION FORM
## Patients Part:

Your Full Name_____

Today's Date: _____/_____/_____ (Day / Month / Year)  Date of Injury: _____/_____/_____ (Day / Month / Year)

**SYMPTOMS*:**

*Grade symptoms at the highest activity level at which you think you could function without significant symptoms, even if you are not actually performing activities at this level.

1. **What is the highest level of activity that you can perform without significant knee pain?**

- ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
- ❑ Strenuous activities like heavy physical work, skiing or tennis
- ❑ Moderate activities like moderate physical work, running or jogging
- ❑ Light activities like walking, housework or yard work
- ❑ Unable to perform any of the above activities due to knee pain

2. **During the past 4 weeks, or since your injury, how often have you had pain?**

| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Never | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | Constant |

3. **If you have pain, how severe is it?**

| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No pain | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | Worst pain imaginable |

4. **During the past 4 weeks, or since your injury, how stiff or swollen was your knee?**

- ❑ Not at all
- ❑ Mildly
- ❑ Moderately
- ❑ Very
- ❑ Extremely

5. **What is the highest level of activity you can perform without significant swelling in your knee?**

- ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
- ❑ Strenuous activities like heavy physical work, skiing or tennis
- ❑ Moderate activities like moderate physical work, running or jogging
- ❑ Light activities like walking, housework, or yard work
- ❑ Unable to perform any of the above activities due to knee swelling

6. **During the past 4 weeks, or since your injury, did your knee lock or catch?**

❑ Yes ❑ No

7. **What is the highest level of activity you can perform without significant giving way in your knee?**

- ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
- ❑ Strenuous activities like heavy physical work, skiing or tennis
- ❑ Moderate activities like moderate physical work, running or jogging
- ❑ Light activities like walking, housework or yard work
- ❑ Unable to perform any of the above activities due to giving way of the knee

## SPORTS ACTIVITIES:

**8. What is the highest level of activity you can participate in on a regular basis?**

- ❑ Very strenuous activities like jumping or pivoting as in basketball or soccer
- ❑ Strenuous activities like heavy physical work, skiing or tennis
- ❑ Moderate activities like moderate physical work, running or jogging
- ❑ Light activities like walking, housework or yard work
- ❑ Unable to perform any of the above activities due to knee

**9. How does your knee affect your ability to:**

| | | Not difficult at all | Minimally difficult | Moderately Difficult | Extremely difficult | Unable to do |
|---|---|---|---|---|---|---|
| a. | Go up stairs | ❑ | ❑ | ❑ | ❑ | ❑ |
| b. | Go down stairs | ❑ | ❑ | ❑ | ❑ | ❑ |
| c. | Kneel on the front of your knee | ❑ | ❑ | ❑ | ❑ | ❑ |
| d. | Squat | ❑ | ❑ | ❑ | ❑ | ❑ |
| e. | Sit with your knee bent | ❑ | ❑ | ❑ | ❑ | ❑ |
| f. | Rise from a chair | ❑ | ❑ | ❑ | ❑ | ❑ |
| g. | Run straight ahead | ❑ | ❑ | ❑ | ❑ | ❑ |
| h. | Jump and land on your involved leg | ❑ | ❑ | ❑ | ❑ | ❑ |
| i. | Stop and start quickly | ❑ | ❑ | ❑ | ❑ | ❑ |

## FUNCTION:

**10. How would you rate the function of your knee on a scale of 0 to 10 with 10 being normal, excellent function and 0 being the inability to perform any of your usual daily activities which may include sports?**

FUNCTION PRIOR TO YOUR KNEE INJURY:

Cannot perform daily activities — No limitation

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |

CURRENT FUNCTION OF YOUR KNEE:

Cannot perform daily activities — No limitation

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |

## SCORING INSTRUCTIONS FOR THE 2000 IKDC SUBJECTIVE KNEE EVALUATION FORM

Several methods of scoring the IKDC Subjective Knee Evaluation Form were investigated. The results indicated that summing the scores for each item performed as well as more sophisticated scoring methods.

The responses to each item are scored using an ordinal method such that a score of 1 is given to responses that represent the lowest level of function or highest level of symptoms. For example, item 1, which is related to the highest level of activity without significant pain is scored by assigning a score of 1 to the response "Unable to Perform Any of the Above Activities Due to Knee" and a score of 5 to the response "Very strenuous activities like jumping or pivoting as in basketball or soccer". For item 2, which is related to the frequency of pain over the past 4 weeks, the response "Constant" is assigned a score of 1 and "Never" is assigned a score of 11.

The IKDC Subjective Knee Evaluation Form is scored by summing the scores for the individual items and then transforming the score to a scale that ranges from 0 to 100. **Note**: The response to item 10 "Function Prior to Knee Injury" is not included in the overall score. The steps to score the IKDC Subjective Knee Evaluation Form are as follows:

1. Assign a score to the individual's response for each item, such that lowest score represents the lowest level of function or highest level of symptoms.
2. Calculate the raw score by summing the responses to all items with the exception of the response to item 10 "Function Prior to Your Knee Injury"
3. Transform the raw score to a 0 to 100 scale as follows:

$$\text{IKDC Score} = \left[\frac{\text{Raw Score - Lowest Possible Score}}{\text{Range of Scores}}\right] \text{x} 100$$

Where the lowest possible score is 18 and the range of possible scores is 87. Thus, if the sum of scores for the 18 items is 60, the IKDC Score would be calculated as follows:

$$\text{IKDC Score} = \left[\frac{60 - 18}{87}\right] \text{x} 100$$

$$\text{IKDC Score} = 48.3$$

The transformed score is interpreted as a measure of function such that higher scores represent higher levels of function and lower levels of symptoms. A score of 100 is interpreted to mean no limitation with activities of daily living or sports activities and the absence of symptoms.

The IKDC Subjective Knee Score can still be calculated if there are missing data, as long as there are responses to at least 90% of the items (i.e. responses have been provided for at least 16 items). To calculate the raw IKDC score when there are missing data, substitute the average score of the items that have been answered for the missing item score(s). Once the raw IKDC score has been calculated, it is transformed to the IKDC Subjective Knee Score as described above.

# ICRS KNEE HISTORY REGISTRATION-PREVIOUS SURGERY

## Surgeons part

Type of surgery: Check all that apply
Meniscal surgery:

**Medial meniscal surgery :**
Partial resection___ Subtotal resection__
Meniscal suture___
Meniscal Transplant___
Open___Arthroscop___

**Lateral Meniscal Surgery**
Partial resection___ Subtotal resection__
Meniscal Suture___
Meniscal Transplant___
Open___Arthroscop__

**Ligament Surgery:**
ACL repair__Intraarticular __ Extraarticular___
PCL-repair__Intraarticular___Extraarticular___
Medial-___Lateral-Collateral-ligament reconstruction___

Type of graft:
Patella-tendon__ Ipsilateral__Contralateral__
Single hamstrings-graft___
2 bundle hamstrings-graft___
4 bundle hamstrings-graft___
Quadriceps-graft___
Allograft___
Other___

**Extensor Mechanism surgery:**
Patella tendon repair___ Quadriceps-tendon repair___

**Patellofemoral surgery:**
*Soft tissue realignement:*
Medial imbrication___ Lateral release___
*Bone realignement:*
Tibial tubercle transfer:
Proximal__Distal__Medial__Lateral__Anterior__
Trochlear plasty__
Patellectomy__

Cartilage resurfacing and reconstructive surgery:
Debridement (shaving of fibrillated cartile and cartilage flaps) ____
Abrasion arthroplast ____
Microfracture ____
Subchondral drilling ____
Carbon fibre resurfacing ____
Osteochondral allograft ____
Multiple osteochondral autologous grafts ____
Periosteal resurfacing ____
Perichondral resurfacing ____
Autologous chondrocyte implantation + periosteum ____
Autologous chondrocyte implantation with membrane ____
Other type of technique: _____ ____

## Surgeons part

Osteotomy: Tibia____Femur____ Varus____Valgus_____

**Imaging techniques:**

Plain x-rays:_____ Varus-angle_____Valgus-angle_____

CT____ CT-arthrography____ MRI____ Scintigraphy_____

**Findings:**

Articular cartilage appearance:_____

_____

Bone:_____ _____

_____

Ligaments:_____ _____

_____

Menisci:_____ _____

_____

# 2000 IKDC KNEE Examination Form

## Surgeons part

**Patient Name :**_____ **Date of Birth:** ____/_____/_____ (Day / Month / Year)

**Gender:** ? F ? M **Age :**_____ **Date of Examination:**_____/_____/_____ (Day / Month / Year)

| | | | | |
|---|---|---|---|---|
| **Generalized Laxity:** | ?tight | ?normal | ?lax | |
| **Alignment:** | ?obvious varus | ?normal | ?obvious valgus | |
| **Patella Position:** | ?obvious baja | ?normal | ?obvious alta | |
| **Patella Subluxation/Dislocation:** | ?centered | ?subluxable | ?subluxed | ?dislocated |
| **Range of Motion (Ext/Flex):** | Index Side: passive_____/_____/_____ | | active_____/_____/_____ | |
| | Opposite Side: passive_____/_____/_____ | | active_____/_____/_____ | |

| SEVEN GROUPS | FOUR GRADES A Normal | B Nearly Normal | C Abnormal | D Severely Abnormal | *Group Grade A | B | C | D |
|---|---|---|---|---|---|---|---|---|
| **1. Effusion** | ? None | ? Mild | ? Moderate | ? Severe | ? | ? | ? | ? |
| **2. Passive Motion Deficit** | | | | | | | | |
| ΔLack of extension | ? <3° | ? 3 to 5° | ? 6 to 10° | ? >10° | | | | |
| ΔLack of flexion | ? 0 to 5° | ? 6 to 15° | ? 16 to 25° | ? >25° | ? | ? | ? | ? |
| **3. Ligament Examination** (manual, instrumented, x-ray) | | | | | | | | |
| ΔLachman (25° flex) (134N) | ? -1 to 2mm | ? 3 to 5mm(1+)<br>? <-1 to –3 | ? 6 to 10mm(2+)<br>? <-3 stiff | ? >10mm(3+) | | | | |
| ΔLachman (25° flex) manual max | ? -1 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| Anterior endpoint: | ? firm | | ? soft | | | | | |
| ΔTotal AP Translation (25° flex) | ? 0 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| ΔTotal AP Translation (70° flex) | ? 0 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| ΔPosterior Drawer Test (70° flex) | ? 0 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| ΔMed Joint Opening (20° flex/valgus rot) | ? 0 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| ΔLat Joint Opening (20° flex/varus rot) | ? 0 to 2mm | ? 3 to 5mm | ? 6 to 10mm | ? >10mm | | | | |
| ΔExternal Rotation Test (30° flex prone) | ? <5° | ? 6 to 10° | ? 11 to 19° | ? >20° | | | | |
| ΔExternal Rotation Test (90° flex prone) | ? <5° | ? 6 to 10° | ? 11 to 19° | ? >20° | | | | |
| ΔPivot Shift | ? equal | ? +glide | ? ++(clunk) | ? +++(gross) | | | | |
| ΔReverse Pivot Shift | ? equal | ? glide | ? gross | ? marked | | | | |
| | | | | | ? | ? | ? | ? |
| **4. Compartment Findings** | | | crepitation with | | | | | |
| ΔCrepitus Ant. Compartment | ? none | ? moderate | ? mild pain | ? >mild pain | | | | |
| ΔCrepitus Med. Compartment | ? none | ? moderate | ? mild pain | ? >mild pain | | | | |
| ΔCrepitus Lat. Compartment | ? none | ? moderate | ? mild pain | ? >mild pain | | | | |
| **5. Harvest Site Pathology** | ? none | ? mild | ? moderate | ? severe | | | | |
| **6. X-ray Findings** | | | | | | | | |
| Med. Joint Space | ? none | ? mild | ? moderate | ? severe | | | | |
| Lat. Joint Space | ? none | ? mild | ? moderate | ? severe | | | | |
| Patellofemoral | ? none | ? mild | ? moderate | ?severe | | | | |
| Ant. Joint Space (sagittal) | ? none | ? mild | ? moderate | ? severe | | | | |
| Post. Joint Space (sagittal) | ? none | ? mild | ? moderate | ? severe | | | | |
| **7. Functional Test** | | | | | | | | |
| One Leg Hop (% of opposite side) | ? ≥90% | ? 89 to 76% | ? 75 to 50% | ? <50% | | | | |
| ****Final Evaluation** | | | | | ? | ? | ? | ? |

* Group grade: The lowest grade within a group determines the group grade

** Final evaluation: the worst group grade determines the final evaluation for acute and subacute patients. For chronic patients compare preoperative and postoperative evaluations. In a final evaluation only the first 3 groups are evaluated but all groups must be documented. Δ Difference in involved knee compared to normal or what is assumed to be normal.

**IKDC COMMITTEE AOSSM:** Anderson, A., Bergfeld, J., Boland, A. Dye, S., Feagin, J., Harner, C. Mohtadi, N. Richmond, J. Shelbourne, D., Terry, G. ESSKA: Staubli, H., Hefti, F., Hoher, J., Jacob, R., Mueller, W., Neyret, P. APOSSM: Chan, K., Kurosaka, M.

## INSTRUCTIONS FOR THE 2000 IKDC KNEE EXAMINATION FORM

The Knee Examination Form contains items that fall into one of seven measurement domains. However, only the first three of these domains are graded. The seven domains assessed by the Knee Examination Form are:

1. *Effusion*
   An effusion is assessed by ballotting the knee. A fluid wave (less than 25 cc) is graded mild, easily ballotteable fluid – moderate (25-60 cc), and a tense knee secondary to effusion (greater than 60 cc) is rated severe.

2. *Passive Motion Deficit*
   Passive range of motion is measured with a gonimeter and recorded on the form for the index side and opposite or normal side. Record values for zero point/hyperextension/flexion (e.g. 10 degrees of hyperextension, 150 degrees of flexion = 10/0/150; 10 degrees of flexion to 150 degrees of flexion = 0/10/150). Extension is compared to that of the normal knee.

3. *Ligament Examination*
   The Lachman test, total AP translation at 70 degrees, and medial and lateral joint opening may be assessed with manual, instrumented or stress x-ray examination. Only one should be graded, preferably a "measured displacement". A force of 134 N (30 lbs) and the maximum manual are recorded in instrumented examination of both knees. Only the measured displacement at the standard force of 134 N is used for grading. The numerical values for the side to side difference are rounded off, and the appropriate box is marked.

   The end point is assessed in the Lachman test. The end point affects the grading when the index knee has 3-5 mm more anterior laxity than the normal knee. In this case, a soft end point results in an abnormal grade rather than a nearly normal grade.

   The 70-degree posterior sag is estimated by comparing the profile of the injured knee to the normal knee and palpating the medial femoral tibia step off. It may be confirmed by noting that contraction of the quadriceps pulls the tibia interiorly.

   The external rotation tests are performed with the patient prone and the knee flexed 30° and 70°.
   Equal external rotational torque is applied to both feet and the degree of external rotation is recorded.

   The pivot shift and reverse pivot shift are performed with the patient supine, with the hip in 10-20 degrees of abduction and the tibia in neutral rotation using either the Losee, Noyes, or Jakob techniques. The greatest subluxation, compared to the normal knee, should be recorded.

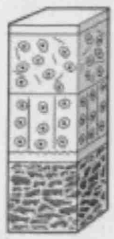
4. *Compartment Findings*
   Patellofemoral crepitation is elicited by extension against slight resistance. Medial and lateral compartment crepitation is elicited by extending the knee from a flexed position with a varus stress and then a valgus stress (i.e., McMurray test). Grading is based on intensity and pain.

5. *Harvest Site Pathology*
   Note tenderness, irritation or numbness at the autograft harvest site.

6. *X-ray Findings*
   A bilateral, double leg PA weightbearing roentgenogram at 35-45 degrees of flexion (tunnel view) is used to evaluate narrowing of the medial and lateral joint spaces. The Merchant view at 45 degrees is used to document patellofemoral narrowing. A mild grade indicates minimal changes (i.e., small osteophytes, slight sclerosis or flattening of the femoral condyle) and narrowing of the joint space which is just detectable. A moderate grade may have those changes and joint space narrowing (e.g., a joint space of 2-4 mm side or up to 50% joint space narrowing). Severe changes include a joint space of less than 2 mm or greater than 50% joint space narrowing.

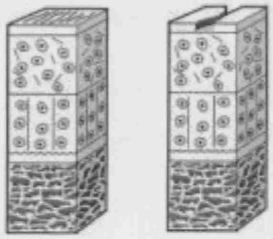
7. *Functional Test*
   The patient is asked to perform a one leg hop for distance on the index and normal side. Three trials for each leg are recorded and averaged. A ratio of the index to normal knee is calculated.

## ICRS Grade 0 - Normal



## ICRS Grade 1 – Nearly Normal

Superficial lesions. Soft indentation (A) and/or superficial fissures and cracks (B)



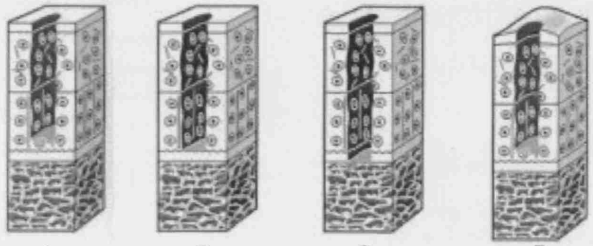
A B

## ICRS Grade 2 – Abnormal

Lesions extending down to <50% of cartilage depth

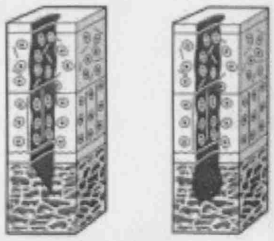
## ICRS Grade 3 – Severely Abnormal

Cartilage defects extending down >50% of cartilage depth (A) as well as down to calcified layer (B) and down to but not through the subchondral bone (C). Blisters are included in this Grade (D)

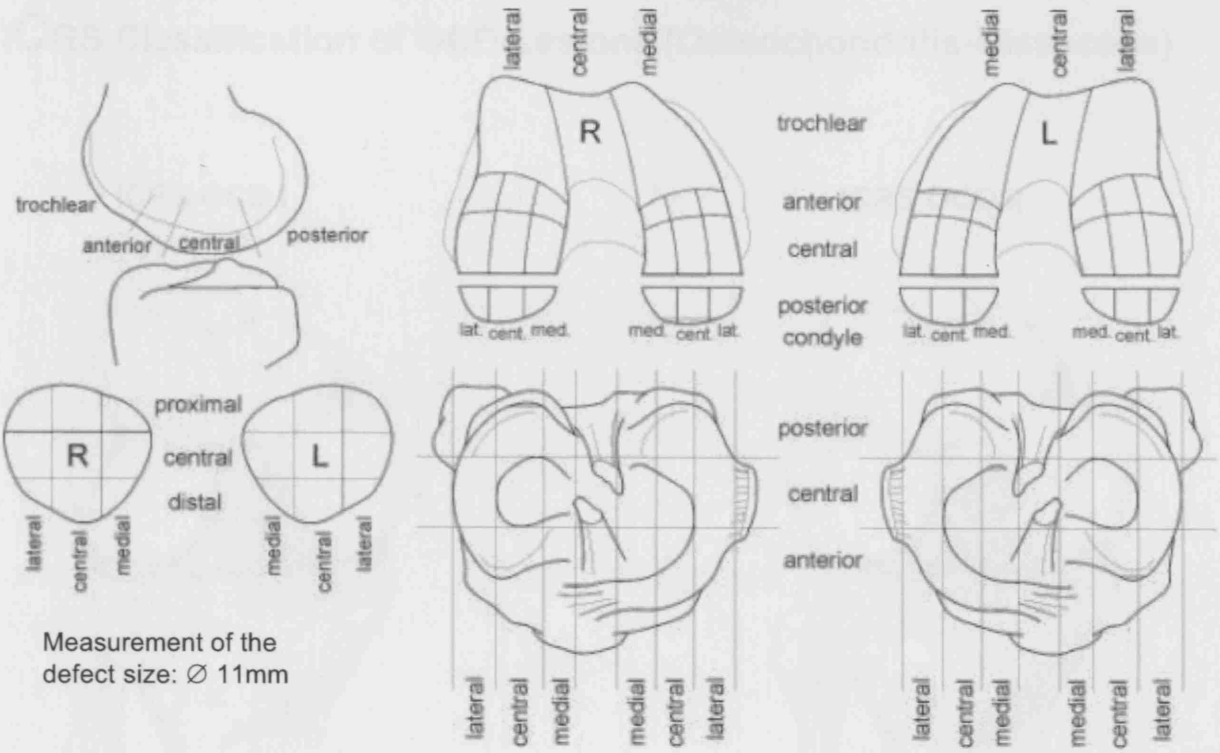


A B C D

## ICRS Grade 4 – Severely Abnormal



A B




Measurement of the defect size: ∅ 11mm

**Femur**

First lesion

Second lesion

| | | | | |
|---|---|---|---|---|
| Side | Right | Left | | |
| Condyle | medial | lateral | | |
| Sagital plane | trochlear | anterior | central | posterior |
| Frontal plane | lateral | central | medial | |

*Cartilage lesion (Grade) (*)*
*Defect size pre-debridement* mm
*Defect size post-debridement* mm

**Tibia**

First lesion

Second lesion

| | | | |
|---|---|---|---|
| Side | Right | Left | |
| Plateau | medial | lateral | |
| Sagital plane | anterior | central | posterior |
| Frontal plane | lateral | central | medial |

*Cartilage lesion (Grade) (*)*
*Defect size pre-debridement* mm
*Defect size post-debridement* mm

**Patella**

First lesion

Second lesion

| | | | |
|---|---|---|---|
| Side | Right | Left | |
| Sagital plane | distal | central | proximal |
| Frontal plane | lateral | central | medial |

*Cartilage lesion (Grade) (*)*
*Defect size pre-debridement* mm
*Defect size post-debridement* mm

**Diagnosis:** Traumatic cartilage lesion OD OA AVN Others

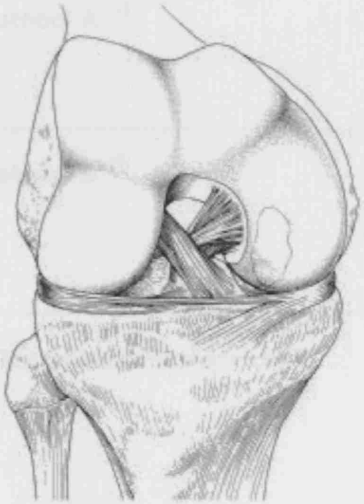
**Biopsy/Osteochondral Plugs:** Location: Number of Plugs: Diameter of Plugs:

**Treatment:** Shaving Drilling Others:
Mosaic-Plasty Microfracture
Autologous Chondrocyte Implantation (ACI)

**Notes:**

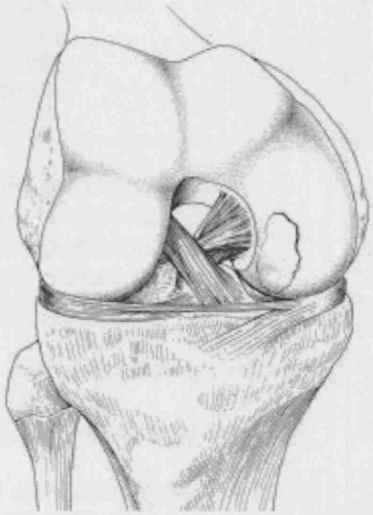
# ICRS Classification of OCD-Lesions (Osteochondritis-Dissecans)

## ICRS OCD I



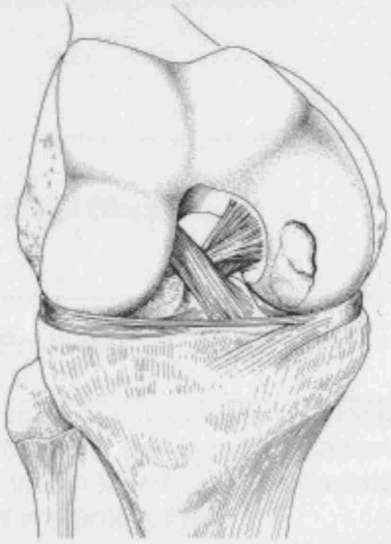
Stable, continuity: Softened area covered by intact cartilage.

## ICRS OCD II



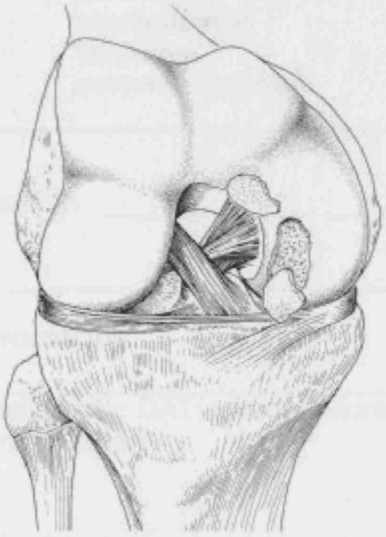
Partial discontinuity, stable on probing

## ICRS OCD III



Complete discontinuity, "dead in situ", not dislocated.

## ICRS OCD IV



Dislocated fragment, loose within the bed or empty defect.> 10mm in depth is B-subgroup

# CARTILAGE REPAIR ASSESSMENT

| Criteria | Points | |
|---|---|---|
| **Degree of Defect Repair**<br>**I Protocol A** [1] | * In level with surrounding cartilage<br>* 75% repair of defect depth<br>* 50% repair of defect depth<br>* 25% repair of defect depth<br>* 0% repair of defect depth | 4<br>3<br>2<br>1<br>0 |
| **I Protocol B** [2] | * 100% survival of initially grafted surface<br>* 75% survival of initially grafted surface<br>* 50% survival of initially grafted surface<br>* 25% survival of initially grafted surface<br>* 0% (plugs are lost or broken) | 4<br>3<br>2<br>1<br>0 |
| **II Integration to Border zone** | * Complete integration with surrounding cartilage<br>* Demarcating border < 1mm<br>* 3/4 of graft integrated, 1/4 with a notable border >1mm width<br>* 1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border > 1mm<br>* From no contact to 1/4 of graft integrated with surrounding cartilage | 4<br>3<br>2<br>1<br>0 |
| **III Macroscopic Appearance** | * Intact smooth surface<br>* Fibrillated surface<br>* Small, scattered fissures or cracs<br>* Several, small or few but large fissures<br>* Total degeneration of grafted area | 4<br>3<br>2<br>1<br>0 |
| **Overall Repair Assessment** | **Grade I** **normal** **12 P**<br>**Grade II** **nearly normal** **11-8 P**<br>**Grade III** **abnormal** **7-4 P**<br>**Grade IV** **severely abnormal** **3-1 P** | |

**Cartilage Biopsy** **Location** _____

| (1) Protocol A: | (2) Protocol B: |
|---|---|
| autologous chondrocyte implantation (ACI); periosteal or perichondrial transplantation; subchondral drilling; microfracturing; carbon fibre implants; others: | Mossaicplasty; OAT; osteochondral allografts; others: |

# Appendix D – PUBLICATIONS ARISING FROM THESIS