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Direct Use of Resorbable Collagen-Based Beads for Cell Delivery in Tissue Engineering and Cell Therapy Applications

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1. Introduction

Tissue engineering and cell therapy represent significant emerging technologies in medicine. Tissue engineering is, "the persuasion of the body to heal itself, through the delivery to the appropriate sites of molecular signals, cells and supporting structures" (Williams, 1999). Various approaches have been described which are labelled tissue engineering, which form a continuum from complex regeneration of scaffold-based tissues to more simple cell-based therapies, where these supporting structures are usually absent or are present in the form of gels simply to aid cell delivery or encapsulation. In tissue engineering the implant to be delivered may frequently be a near mature functional product or a cell-seeded tissue scaffold, both derived in a bioreactor (Haycock, 2011). For cell-based therapies, a bioreactor is not necessary but may be used to amplify the cell numbers for the therapy. Indeed, a key element for both approaches is that they normally require significant expansion of the replacement cells, which have typically been derived in low numbers from small biopsy samples (Brittberg et al., 1994; Chiang et al., 2005; Thissen et al., 2005). A consequence of this expansion is that there can be a significant loss in the quality of cells available for the procedure.

In many cases, monolayer cell culture has been the method of choice for cell expansion (Brittberg, 1999; Brittberg et al., 1994; Henderson et al., 2007). However, while this method is well established and simple it has problems. One of these is that certain cells may de-differentiate and lose their phenotype and biochemical characteristics (Arterburn et al., 1995; Mallein-Gerin et al., 1991). For example, in extended culture, chondrocytes lose their rounded morphology (Fig. 1A) and develop an extended, fibroblast like appearance (Fig. 1B). Also, accompanying this morphological change, matrix biosynthesis switches from type II collagen to type I collagen (Aulthouse et al., 1989; Lefebvre et al., 1990; von der Mark et al., 1977). It is clear that the collagen produced changes from type II collagen (Fig. 2A), which is the characteristic collagen of articular, hyaline cartilage (Mayne, 1989; Miller & Matukas, 1969) to type I collagen (Fig. 2B), which is a mechanically-inferior fibrocartilage (Chiang et al., 2005; Wegener et al., 2009). Type I collagen is the principal collagen of many tissues,

including skin, bone, ligament and tendons. It is also present in poorly functioning fibrocartilage which can be observed in some arthritic conditions (Aulthouse et al., 1989; Lefebvre et al., 1990; von der Mark et al., 1977). Depending on the cell type, variations in media or other conditions can potentially be used to extend the control on phenotype and biochemical characteristics. For example, lowering of the amount of oxygen has been suggested for control of chondrocyte phenotype in monolayer cultures (Malda et al., 2003), but the positive effect may be limited, possibly to around 10 days (Malda et al., 2003, Oesser & Seifert, 2003), which is less time than would typically be needed for cell expansion for cell therapy applications (Brittberg et al., 1994). Stem cells, especially embryonic stem cells have particular difficulties in maintaining stemness in culture and current trends are aimed at developing specialised media or scaffold-based bioreactors to expand these types of cells.

Chondrocytes have been a cell type of particular interest as they are the cell used in a major cell therapy application, autologous chondrocyte implantation (ACI). The development of ACI has proved to be a particularly useful innovation for certain types of cartilage defects (Brittberg et al., 1994; Brittberg, 1999), and is now an established cell-based therapy for regeneration of certain types of articular cartilage damage. Defects of articular cartilage in the knee do not have the capacity to self repair (Hippocrates, cited by Hunter, 1742-1743). A range of surgical techniques have been proposed for repair of damage and pain relief. These include debridement, abrasion, mosaicplasty and microfracture (Bedi et al., 2010). A range of grafting options have also recently been developed, including osteochondral autografts and allografts and periosteal grafting (Hunziker, 2001; Shah et al., 2007). In ACI a cartilage biopsy sample is taken from the patient that yields low numbers of chondrocytes, which then must be expanded several fold to give sufficient cells for the regeneration procedure. In ACI, the cell expansion takes place in monolayer culture and may take 21 days or longer (Brittberg et al., 1994; Chiang et al., 2005; Henderson et al., 2005). As a consequence, the majority, if not all, of the implanted cells are likely to have lost their chondrocyte characteristics. This compromises the regeneration of a true hyaline cartilage after implant (Brittberg, 1999; Hunziker, 2001; Temenoff & Mikos, 2000). Several other variants on this chondrocyte-based techniques have subsequently been proposed to give tissue regeneration constructs that are placed and secured in the defect (Bartlett et al., 2005; Marlovits et al., 2005). These methods, including ACI, provide methods for pain relief and extend the time period before a total knee reconstruction is required.

There are clear deficiencies in using monolayer culture for cell expansion. An alternative culture system that has been developed is based on spinner culture for cells in suspension (McLimans et al., 1957), which was subsequently adapted to use beads as cell microcarriers (van Wezel, 1967). Microcarriers have been shown to provide clear advantages for cell expansion, as the rate of cell expansion can be considerably greater, and for chondrocytes, the cell phenotype, including the biochemical characteristics, is substantially, if not fully retained (Fronzo et al., 1996; Malda et al., 2003, 2006). Compared to monolayer culture, the mechanical effects that are present in spinner culture may lead to positive effects on certain cell types (Freeman et al., 1994). A wide range of microcarriers have been developed, predominantly based on synthetic materials, which provide a wide range of surface chemistries, such as negatively and positively charged and capable of further modification to introduce biological functions (Jacobson & Ryan, 1982).

The benefits of using spinner culture for chondrocyte expansion for potential application to ACI were initially demonstrated using non-resorbable beads (Fronzo et al., 1996; Lahiji et al., 2000), and spinner culture was shown to be significantly superior to monolayer culture,

with excellent proliferation rates achieved while the cells retained their phenotype (Fronzosa et al., 1996). In addition, chondrocytes cultured on a variety of bead types in spinner culture systems have a greater ability to re-differentiate to the differentiated phenotype (Lee et al., 2011; Malda et al., 2003).

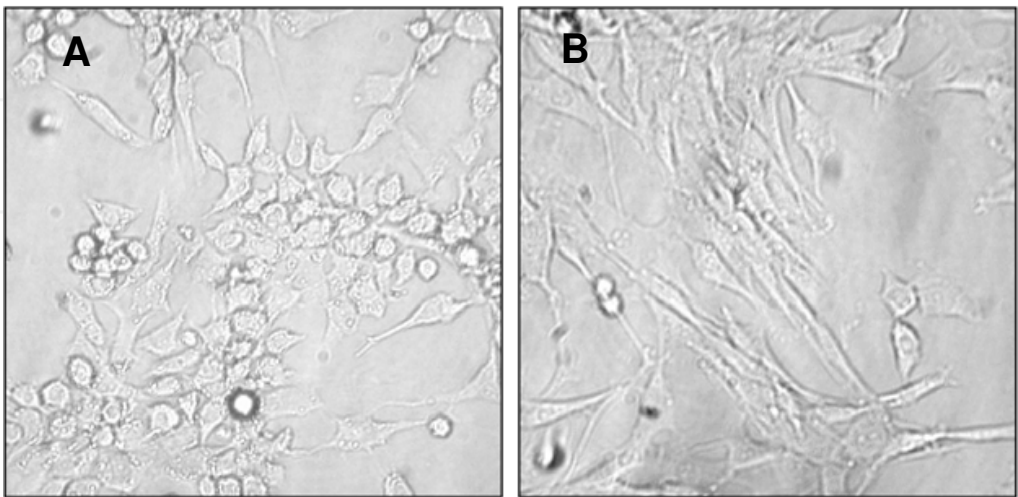


Fig. 1. De-differentiation of chondrocytes in monolayer culture to fibroblast-like phenotype. (A) Cells after 3 days in culture, starting to show some elongated characteristics, (B) Cells after 6 days in culture, showing a predominately elongated, fibroblast-like shape

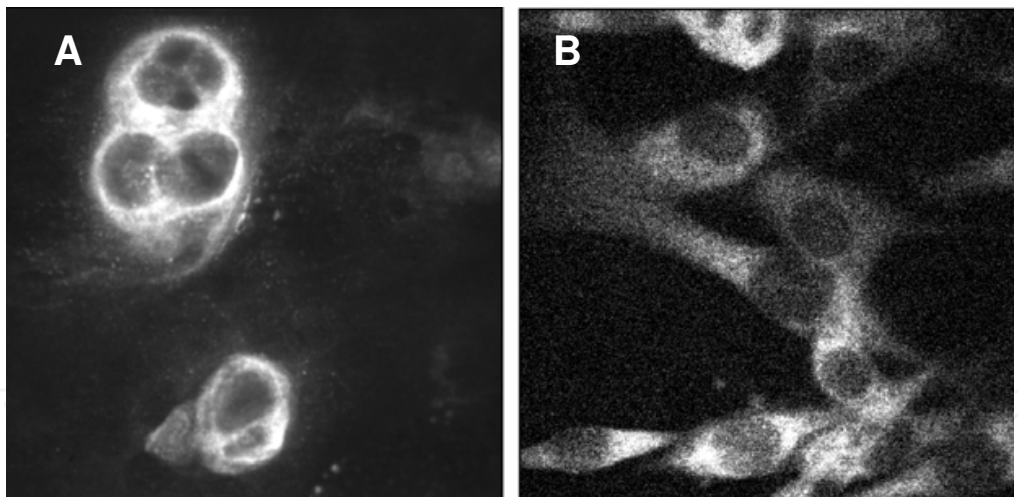


Fig. 2. De-differentiation of chondrocytes in monolayer culture to fibroblast-like phenotype, with collagen production identified by immune-histological staining. (A) Immunostaining for type II collagen production by cells after 3 days in culture. (B) Immunostaining for type I collagen production by cells after 6 days in culture

Various approaches are available for making beads that would potentially be suitable for cell therapy, and several types, for example Cytodex™ beads (Fig. 3A), are commercially available for cell culture. In some cases the beads are biodegradable, for example, resorbable synthetic polymeric (PLGA) beads (Fig. 3B) have been described, including those with surface modifications to enhance cell attachment (Chen et al., 2006; Hong et al., 2008; Thissen et al., 2005).

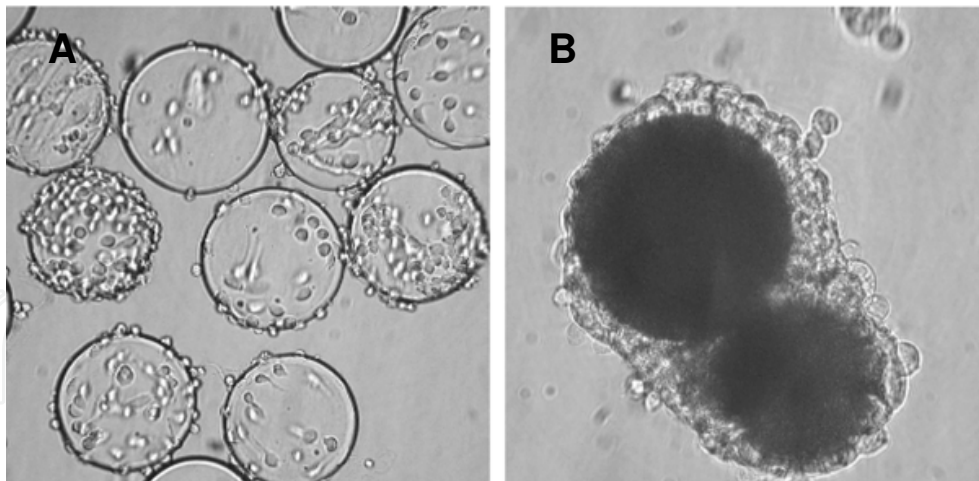


Fig. 3. Chondrocytes in culture on synthetic beads. (A) Culture for 6 days on Cytodex™ beads coated by absorption with type II collagen, showing retention of a round cell morphology. (B) Culture for 12 days on PLGA beads (Thissen et al., 2005) coated by absorption with type II collagen, showing proliferation of cells with rounded morphology

Natural, extracellular matrix tissues can also be used to make beads for spinner culture, and some, for example highly crosslinked gelatin, Cultispher™, are commercially available. Natural tissue-based beads have a range of advantages compared with synthetic beads, including the presence of natural cell binding and growth factor binding sites, a three dimensional environment including variation in surface topography, in vivo resorption using normal biochemical pathways, and when crosslinking is introduced, control of the resorption rate can be achieved (Glattauer et al., 2010). These beads can often be readily processed for cell isolation; for example, mildly crosslinked gelatin beads can be readily dispersed using trypsin or collagenase allowing easy separation of cells by filtration. A wide range of possible beads has been described, along with various approaches for manufacture. Beads can be made of natural tissue components (Glattauer et al., 2010), including those based on powdered acellular dermis (Cymetra™) (Maloney et al., 2004), porous collagen beads that are formed after removal of an alginate carrier (Tebb et al., 2007), or mildly, <0.5% (Glattauer et al., 2010) or heavily, >>1%, cross-linked gelatin (Tao et al., 2003).

If cells are expanded for cell therapy using an in vivo biodegradable, resorbable bead, then there is no need to isolate the cells from the beads prior to administration (Glattauer et al., 2010; Werkmeister et al., 2006). Inclusion of the resorbable beads as an integral part of the therapy procedure provides the advantage of minimizing the extent of cell handling and eliminating a final trypsin treatment to detach cells from the beads. Delivery of a cell/bead construct is also preferably performed using a gel system so that an appropriate distribution of the material is obtained and the cell/bead construct can be readily administered. A gel delivery system could be a synthetic polymer, preferably one that could be cured in situ to prevent migration after delivery of the cell/bead materials, while maintaining sufficient porosity to allow cell respiration and growth (Adhikari et al., 2010; Gunatillake et al., 2006; Werkmeister et al., 2010). Another good material for a delivery system is collagen. When cold solutions of soluble collagen are warmed to body temperature, 37 °C, the soluble collagen forms into fibrils and a gel then forms (Gross and Kirk, 1958). This gel is biocompatible, and has been the basis for soft tissue augmentation technology (Knapp et al., 1978; Kaplan et al., 1983). Hence, with use of cell/bead constructs, where the cells do not

require final removal after the expansion phase and can be implanted directly as a cell/bead/gel delivery system, the "curing" of the collagen delivery gel will hold the cell/bead constructs in place. In addition, the potential presence of some extracellular matrix (ECM) that had accumulated during culture (Fron dosa et al., 1996; Tebb et al., 2007; Thissen et al., 2005) could assist in retaining the cells at the desired implant location. When resorbable beads are used for cell expansion and then direct delivery of cells, it is possible to design suitable carrier beads with a range of stabilities that matches the implant requirements.

2. Materials and methods

2.1 Preparation of beads

2.1.1 Gelatin beads

Solid gelatin-based beads with cross-linking throughout the bead were produced as previously described (Glattauer et al., 2010). Briefly, 20% w/v porcine A-type gelatin, 270-300 g Bloom (Sigma, St Louis, MO) in 50 mM acetic acid was heated to 50 °C and dispersed at 10% v/v in olive oil at 37 °C by rapid stirring. After 90 min, the emulsion was transferred to 4 °C and stirred for 30 min. Beads were separated from oil by 3 extractions with 0.2% Triton X-100 in phosphate buffered saline, and then cross-linked by addition of selected concentrations of glutaraldehyde (GA) ranging from 0.005% to 0.1%. Cross-linked beads were further extracted with 0.2% Triton X-100 in phosphate buffered saline, water and finally EtOH. Beads were collected by filtration and freeze dried.

2.1.2 Gelatin/collagen beads

For gelatin/collagen beads containing type I or II collagen, collagen was added to cooled, 37 °C, gelatin to give a 10% w/w collagen to gelatin mixture immediately prior to emulsion formation, as for gelatin only beads. Soluble bovine hide type I collagen and soluble bovine articular cartilage type II collagens were prepared using pepsin digestion (see below).

2.1.3 Collagen beads

Collagen beads, based on co-formation with alginate followed by alginate removal were prepared as previously described (Tebb et al., 2007).

Collagen beads can also be formed by an emulsion method, similar to that used to prepare gelatin beads (see above). Type I collagen in phosphate buffered saline, pH 7.3, at 8 mg/ml and 4 °C was dispersed at 10% v/v in olive oil at 12 °C by rapid stirring. After 15 min, the emulsion was heated quickly to 37 °C and stirred very gently. The collagen was allowed to gel for 3 h at 37 °C. The collagen gel beads were then stabilised by addition of 0.1% w/v glutaraldehyde and were crosslinked overnight. Beads were washed, as above for the gelatin beads, collected and freeze dried, followed by ethylene oxide sterilisation prior to use. The typical size range prior to fractionation, eg: by sieving, was 100-200 µm.

2.1.4 Bone based beads

Powdered bovine bone particles were obtained from Waitaki Biosciences (Christchurch, NZ) and were sieved to give a 70 to 150 µm fraction. For production of demineralised bone (DMB) particles, the bovine bone particles were suspended in either 0.6 M HCl, or 0.5 M EDTA at pH 7.4, and stirred for 16 h at 4°C. After settling, solutions were removed by decanting. Particles were then further extracted each day for 6 days using the same

conditions, before being washed and freeze dried (Glattauer et al., 2010). A 70 to 150 μm fraction was collected by sieving. The loss of Ca was confirmed as previously described (Glattauer et al., 2010).

2.1.5 Endosteal particles

Endosteal particles from bovine bone marrow were prepared from fresh sternum from young calves, ~4 weeks old (Nigro et al., 2010). Briefly, dissected pieces of sternum were crushed in liquid nitrogen and the frozen bone marrow powder was freeze-dried. To decellularise the dried particles, they were suspended in water and mixed vigorously for 30 min, and then allowed to settle. The water was replaced with 4% ethanol/0.1% peracetic acid and mixed vigorously for 2 h, and then allowed to settle. The solution was then replaced and the particles mixed with phosphate buffered saline for 15 min, and then allowed to settle. This wash was repeated and then the settled particles were treated with a cocktail of DNase I (50 U/ml) and RNase A (1 U/ml) in 10 mM Tris-HCl/2.5 mM MgCl_2 /0.5 mM CaCl_2 , pH 7.6 for 24 h at 37 °C, with gentle agitation. The particles were washed with phosphate buffered saline and then water, and then exchanged into 80% v/v ethanol and sieved to provide a fraction between 40 - 230 μm . To remove any residual calcification, particles were treated with 0.6 N HCl for 16 h at 4 °C, washed three times in sterile water and then stored in 80% (v/v) ethanol.

2.1.6 Basement membrane particles

Fresh bovine testis was obtained from an abattoir. After removal of the external membranes, the tissue comprising the seminiferous tubules was sliced into approximately 5 x 5 x 5 mm pieces and was macerated on a stainless steel wire mesh to break the tissue and release cells with continual irrigation by phosphate buffered saline, including a protease inhibitor cocktail. The tubules were retained on the mesh. Samples were suspended in excess phosphate buffered saline containing protease inhibitor cocktail, and washed and collected by settling 3 times. Washed samples were examined by immunohistology to examine the presence of basement membrane components using antibodies to laminin (Sigma) and collagen type IV (Biodesign, ME) following the methods previously described (Glattauer et al., 2007). Basement membrane samples were fragmented further using Ultra Turrax blender (IKA Werke, Germany). The resulting suspension was examined by microscopy to determine the particle size that was present. The particles were observed to have a maximal dimension of 500 μm . The basement membrane particles can be fractionated if required to give particles of the desired size by use of mesh sieves of appropriate size.

All collagen-based particle types were sieved to collect 70 to 150 μm fractions and were sterilised by ethylene oxide, except the seminiferous tubule-derived particles which were sanitised by soaking in 80% EtOH. Bead size distributions were verified by microscopy and image analysis. Unmodified Cytodex™ beads (Amersham Biosciences, Sweden) were used as a synthetic control bead and were sterilised prior to use by autoclaving.

2.2 Culture of chondrocytes

2.2.1 Isolation of chondrocytes

Ovine chondrocytes were isolated from fresh articular cartilage by digestion of 1 mm³ pieces with 10 % trypsin (2 ml/g) (MP Biomedicals), for 1 h at 37 °C, followed by 300 units/ml bacterial collagenase (354 U/ml *Clostridium histolyticum*, type IAS, Sigma, St Louis, MO) and

760 units/ml hyaluronidase (2 ml/ g) (760 U/ml bovine testicular type IV-S, Sigma, St Louis, MO), for 16 h at 37 °C. Cells were collected through a 70 μ m filter, and washed and counted prior to seeding. Human articular chondrocytes were obtained from Edward Keller (Cambrex BioScience, Mount Waverley, Australia), (EK 23-7-02, KN8823) and maintained in complete chondrocyte medium (F12:DMEM, 1:1, containing 10% FCS, plus 1 mM glutamine and 10 mM 2-mercaptoethanol); comparable results were obtained with both the ovine and human cells.

2.2.2 Chondrocyte seeding and culture on beads

Cells were seeded onto the various microcarrier beads and cultured in spinner bottles, with an initial density of 5×10^5 cells/150 mg particles in 50ml DMEM/F12/10% FBS containing 100 μ g/ml penicillin and streptomycin, at 37 °C in 5% CO₂. Cell seeding was performed with initial 25 rpm intermittent stirring for 2 min every 30 min. After 3 h, the stirring speed was increased to 45 rpm, intermittently as above, for 1.5 h, followed by continuous stirring at 15 min intervals at 45, 50, 55 and finally 60 rpm. After 24 h in culture, the efficiency of cell attachment onto the beads was monitored.

2.2.3 Mesenchymal stem cell seeding and culture on particles

Mesenchymal stem cells were obtained and seeded as previously described (Nigro et al., 2010). A settled volume (0.45 ml) of particles was exchanged into 50 ml MEM/20% FCS/5 ng/ml FGF-2 before adding 2.5×10^5 mesenchymal stem cells in a spinner flask. The flask was stirred at 25 rpm for 2 min every 30 min at 37 °C with 5%CO₂. After 24 h, the spinner flask was set to stir at 25 rpm continuously. After 7 days, a sample of the mesenchymal stem cells/particle construct was taken and cell viability was assessed using Calcein-AM. Images were captured with a fluorescence microscope (Olympus).

2.3 Collagen materials

2.3.1 Isolation and preparation of collagen

Type I bovine collagen was isolated and purified from bovine dermis by pepsin digestion (1 mg/ml) in 50 mM acetic acid at 4 °C as previously described (Ramshaw, 1986). Type I collagen was collected by precipitation by 0.7 M NaCl, and was further purified by differential salt precipitation at neutral pH (Trelstad et al., 1976). Type II collagen from bovine articular cartilage was isolated in a similar manner. The purified bovine collagens were dialysed exhaustively against 20 mM acetic acid and then freeze dried and stored at -20 °C.

2.3.2 Preparation of collagen gels

To prepare collagen gels, purified collagen was dissolved at 3 mg/ml in 50 mM acetic acid. Once dissolved, the solution was taken to 1 M acetic acid and sealed in a dialysis bag and held against 1 M acetic acid for 24 h. It was then dialysed exhaustively against water followed by 20 mM acetic acid and then taken to about 25 mg/ml by air evaporation at room temperature. Finally, while still maintaining the seal of the dialysis tube, it was taken to 4 °C and dialysed against water and then phosphate buffered saline, prior to removal from the dialysis bag under sterile conditions in a biohazard cabinet.

2.3.3 Preparation of collagen gel and cell-bead composite plugs

Cells were taken after culture on beads in a spinner culture system. Typically, 14×10^6 cells on 0.5 ml beads were used. This was to mimic the concentration of cells in natural hyaline

cartilage. The beads/cells were incorporated by mixing with 0.4 ml of 20 mg/ml type I collagen gel in phosphate buffered saline containing 0.1 ml of 10x concentration cell culture medium. The mixture was then taken into a syringe and centrifuged briefly at low speed to remove any air bubbles and 200 μ l aliquots dispensed into 96 well tissue culture plate, which was then incubated at 37 °C for 1 h to allow collagen fibril formation and collagen gelation to occur (Gross and Kirk, 1958). The cell-bead-collagen plugs were then transferred into 24 well culture plates, covered with cell culture medium, and kept in culture for up to 21 days. Ascorbate was added daily to a final concentration 50 μ g/ml.

2.4 Evaluation of tissue samples

2.4.1 Histological evaluation

Samples were processed for conventional histology to examine the extent of bead and particle degradation as well as the extent of cellular infiltration into the collagen/bead (particle) plugs. Stains used were standard Haematoxylin and Eosin and Alcian Blue at pH 2.5 and pH 5.8.

2.4.2 Immunohistological evaluation

Immunohistology staining used specific antibodies against collagen types I, II, VI and other extracellular matrix components. Prior to staining with antibodies, cultured cells on beads were washed in warm phosphate buffered saline, and then pre-fixed in ice cold methanol. A range of antibodies was used to assess the type of matrix: goat anti-collagen type II (Southern Biotechnology, Birmingham, AL), goat anti-collagen type I (Southern Biotechnology, Birmingham, AL), mouse monoclonal anti-collagen type VI (1D8-F8/Col6) (Werkmeister et al., 1993) and mouse monoclonal anti-keratan sulphate (5D4, Seikagaku Corp., Tokyo, Japan). Beads were incubated with antibodies for 1 h, washed and then reacted with corresponding FITC-conjugated secondary antibodies, rabbit anti-goat IgG (Southern Biotechnology, Birmingham, AL) or sheep anti-mouse Ig (Silenus, Melbourne, Australia). As controls in all studies, primary antibodies were omitted and replicate slides were stained with only secondary FITC-labeled antibodies.

3. Results and discussion

3.1 Production of collagen-based beads

We have successfully prepared a wide range of biological, collagen-based beads from a variety of starting tissues. These include beads based solely on gelatin, the denatured form of collagen, and on gelatin mixed with non-denatured collagen (Fig. 4) which are readily formed by an emulsion method. Further stabilisation by crosslinking is then used, as otherwise the gelatin beads would not be stable at the 37 °C required for the subsequent cell culture. In the present case, glutaraldehyde has been used as the crosslinking agent. Variation in the extent of the glutaraldehyde crosslinking allows control of the rate of turnover of the beads (Glattauer et al., 2010). A wide range of other crosslinking agents are also possible (Ramshaw et al., 2000) which have also been used successfully in biomedical materials for clinical applications. The gelatin beads formed regular spheres with a reasonably smooth surface (Fig. 4A), although at high power, using SEM, some texture is present (Glattauer et al., 2000). The gelatin beads containing either 10% w/w collagen type I or type II were again uniform spheres, but in this case both showed some surface texture

(Fig. 4C, Fig. 4E). When the beads were dehydrated in EtOH, the gelatin beads retained their spherical shape (Fig. 4B), while the collagen containing beads became irregular in shape (Fig. 4D, Fig. 4F). However, when these beads were re-hydrated for cell culture they generally returned to their previous spherical shape.

Beads can also be made from collagen alone, without the gelatin component, by various approaches. For example, the emulsion approach used for gelatin (Fig. 4A) can be adapted for use with collagen. In this case the collagen solution at neutral pH must be kept cold during the emulsification stage, so as to prevent fibril formation. Once an emulsion has been formed, it is then warmed to 37 °C which causes the collagen to form fibrils (Gross & Kirk, 1958) and then stable gel particles. Like the gelatin beads, these can then be further stabilised by crosslinking using glutaraldehyde or other reagents (Fig. 5).

Alternatively, collagen beads can be formed by the use of a carrier during the bead forming stage, which is subsequently removed. For example (Fig. 6) alginate has been used as an easy and effective carrier system. In this case a mixture of alginate with collagen that contains a high proportion, ~30-40%, of collagen can be formed, and beads are produced by dropwise (aerosol) addition to 1.5% CaCl₂ (Tebb et al., 2007). The CaCl₂ leads to the precipitation of the alginate to form stable beads, and the collagen can be stabilised by fibril formation and crosslinking as required. The alginate phase can be removed by treatment with Na citrate, giving a collagen only bead (Tebb et al., 2007). An advantage of this method is that the collagen beads are quite porous, and cell infiltration readily occurs (Tebb et al., 2007). Other materials could be used as carriers, including gelatin and other proteins. The collagen triple-helix structure is generally highly stable to proteolytic action, and pepsin treatment is often used during its preparation (Miller & Rhodes, 1982). So a protein carrier, such as gelatin, can be removed by proteolysis, leaving porous collagen beads.

Collagen beads can also be formed directly from tissue. For example, Cymetra™ is prepared from acellular human dermis by fragmentation (Maloney et al., 2004) and is used clinically for tissue augmentation and equivalent procedures. The particles can also be used readily as a cell growth substrate.

An alternate source of collagen beads or particles is from natural collagen containing tissues. The principle protein component of bone is type I collagen. Hence bone particles themselves may present surface collagen to cells to enhance binding (Fig. 7A). Bone particles can be refined using demineralisation, for example with EDTA or HCl, to produce beads enriched of collagen (Glattauer et al., 2010) (Fig. 6B). There are also low levels of growth factors and other biological macromolecules that can add to the effectiveness of the collagen particles in promoting cell adhesion and proliferation. The type and quantity of these molecules may vary depending on the method used to demineralise the tissue and may vary between batches (Wildermann et al., 2007). However, when chondrocytes were grown on particles prepared by both these methods no significant difference was seen in the proliferation rate for the cells (Glattauer et al., 2010). The bone and demineralised bone particles differ significantly from the previous gelatin and collagen beads in that they are irregular in shape, have an irregular surface topography, and do not need additional crosslinking as they are already fully stabilised by the naturally occurring collagen crosslinks. Any soluble collagen would be removed during the demineralisation process. The demineralised bone particles are preferred for spinner culture as they are more bouyant and hence easier to use. Whereas the area of smooth spherical beads in an aliquot, such as synthetic beads or gelatin beads, can be readily calculated from the size distribution of the beads, this is more difficult for irregular shaped beads, and beads with variations in surface topography.

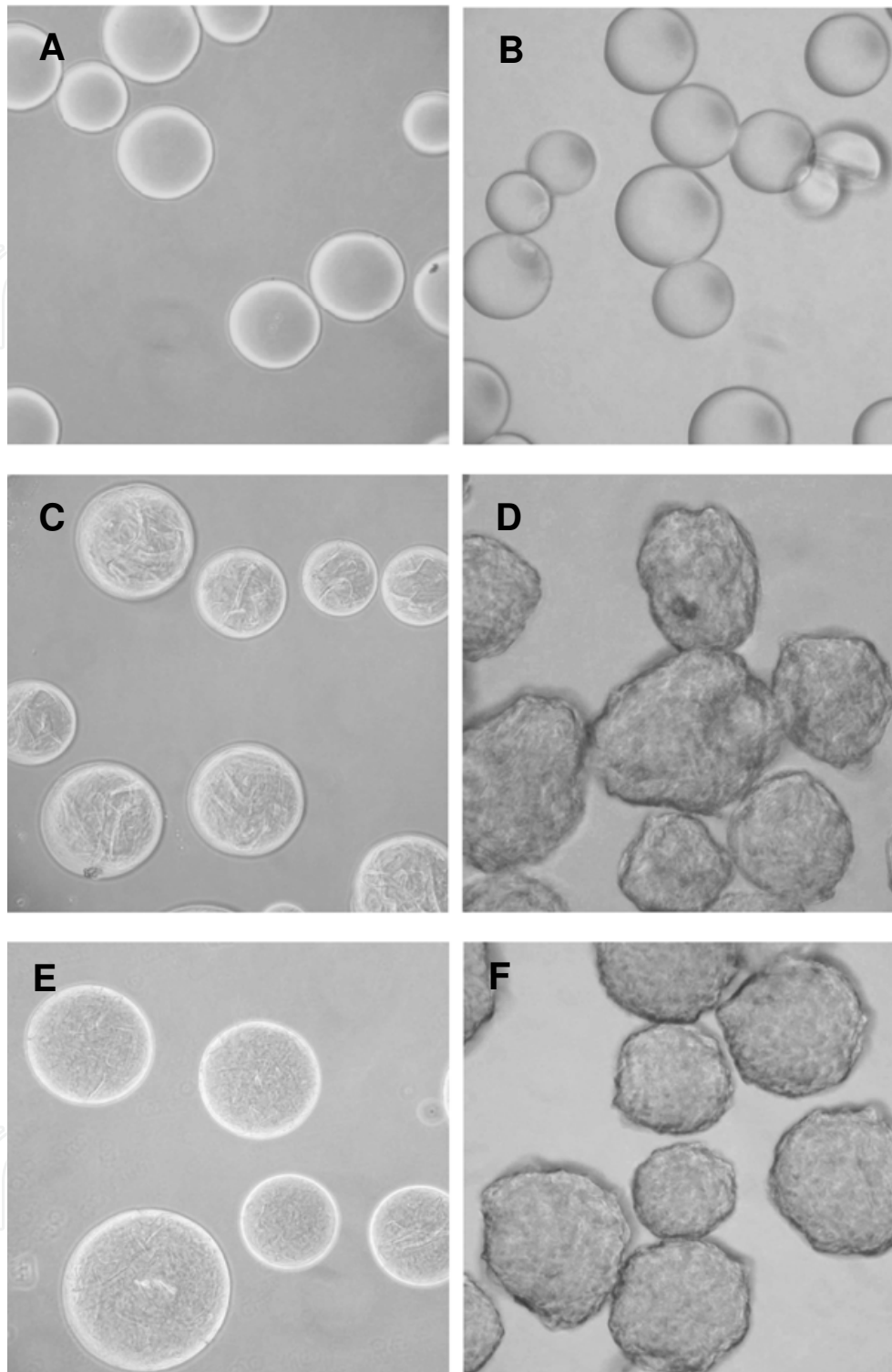


Fig. 4. Production of gelatin and gelatin/collagen beads. Beads in phosphate buffered saline (A, C, E) and after dehydration in EtOH (B, D, F). After rehydration, these beads returned to their original size and shape. (A, B) Gelatin beads, average hydrated bead size prior to fractionation, 106 ± 20 μm . (C, D) Gelatin/10% w/w type I collagen beads, average hydrated bead size prior to fractionation, 169 ± 53 μm . (E, F) Gelatin/10% w/w type II collagen beads, average hydrated bead size prior to fractionation, 159 ± 53 μm

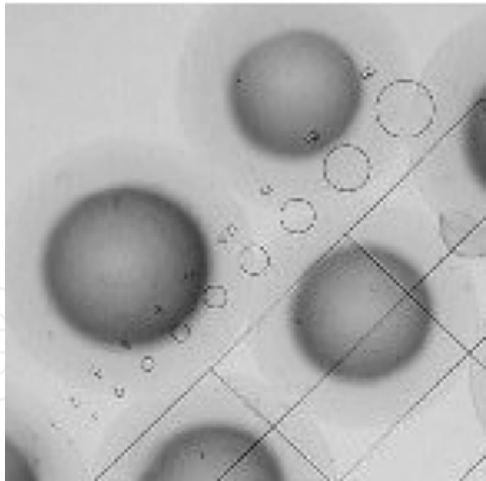


Fig. 5. Collagen beads prepared by an emulsion method

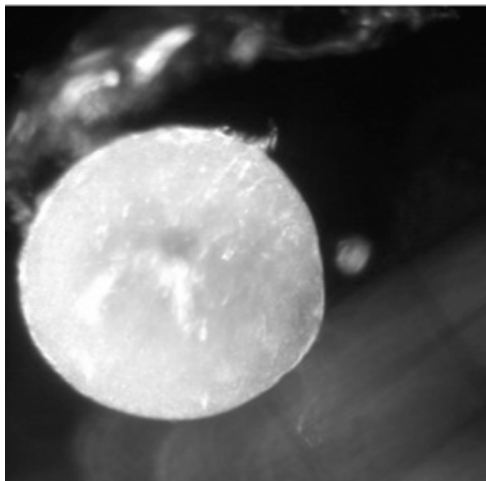


Fig. 6. Production of type II collagen beads using an alginate carrier (Tebb et al., 2007), with type II collagen at 6.6 mg/ ml and alginate at 0.3% during initial bead formation. Bead size ~ 0.7 mm

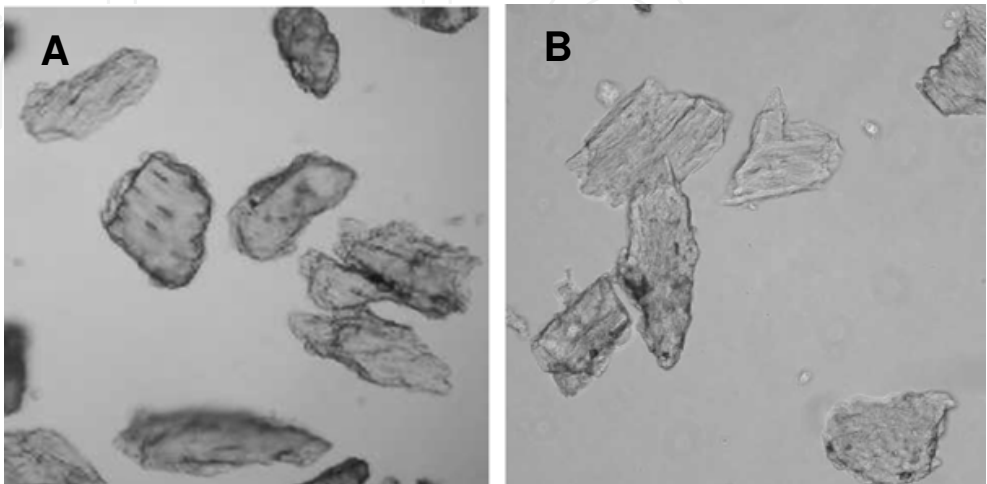


Fig. 7. Examples of (A) bone particles, and (B) demineralised bone particles

The gelatin, collagen and bone particles are all based principally on type I collagen. Other collagens are abundant in different tissues. For example, type II collagen is the collagen found in normal hyaline cartilage (Mayne, 1989; Miller and Rhodes, 1982) and which could be used to make type II collagen particles by tissue fragmentation and further processing, if required, to remove other components such as proteoglycan. Similarly, type III collagen is a major component of blood vessels, along with type I collagen and elastin (Hanson & Bentley, 1983; McCloskey and Cleary, 1974). Hence, particles rich in type III collagen could be prepared by processing arterial tissue. Type IV collagen is the major collagen component of basement membranes. Basement membranes are unique, highly organised supportive sheet-like structures in the extracellular matrix that are formed at the interface between parenchymal cells and their surrounding tissues (Yurchenco et al., 2004). There are different types of basement membranes in the body. Some act as a tissue boundary where certain cells can attach; some can act as filters with selective permeability, and some support very selective cellular differentiation of a number of different cell types, including stem cells. The basement membrane comprises a range of specific macromolecular components. As well as type IV collagen, which may be important for mechanical properties (Yurchenco et al., 2004), the other components are laminin, nidogen and heparan sulphate proteoglycans, such as perlecan, which are believed to interact in a highly ordered manner with type IV collagen (Yurchenco et al., 2004).

Thus, we have shown that by using natural tissues from different origins, it is also possible to make other tissue derived particles. By having different protein and other macromolecules present, these have the potential to provide different interactions with various cell types. An example is the production of particles from the endosteum of bone (Fig. 8) (Nigro et al., 2010). This tissue is proximal to the location of haematopoietic stem cells (Haylock et al., 2007) and so may be useful in the proliferation of this cell type. The particle composition is distinct; examination of the composition using immunohistology showed that they contain perlecan and some laminin, suggesting similarity to a basement membrane, but collagen type IV was not detected (Nigro et al., 2010).

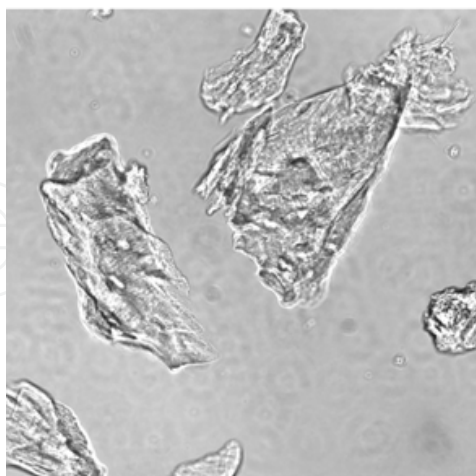


Fig. 8. Decalcified endosteal particles

Basement membrane particles can also be formed from a range of other tissues, including placenta, muscle and kidney (Werkmeister et al., 2006). In the present example, particles have been prepared from seminiferous tubules. The tubules are readily isolated from testis tissue (Fig. 9A), and immunohistological analysis has shown that the major components of

collagen IV (data not shown) and laminin (Fig. 9B) have been retained during the processing. Further fragmentation can be used to prepare particles (Fig. 9C). The composition of this basement membrane is distinct (Glattauer et al., 2007) showing the presence of various laminin types based on the positive staining for the $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ chain of collagen type IV and the positive staining of various laminin chains, including the $\alpha 2$ and $\beta 2$ chains. This composition is distinct from that of the commercial basement membrane material, Matrigel™, which is based on $(\alpha 1)_2\alpha 2$ type IV collagen and $\alpha 1\beta 1\gamma 1$ laminin (laminin 111)(Kleinman & Martin, 2005). Matrigel™ has been used extensively for addressing cell attachment so a different composition basement membrane may provide a useful alternative option for cell culture.

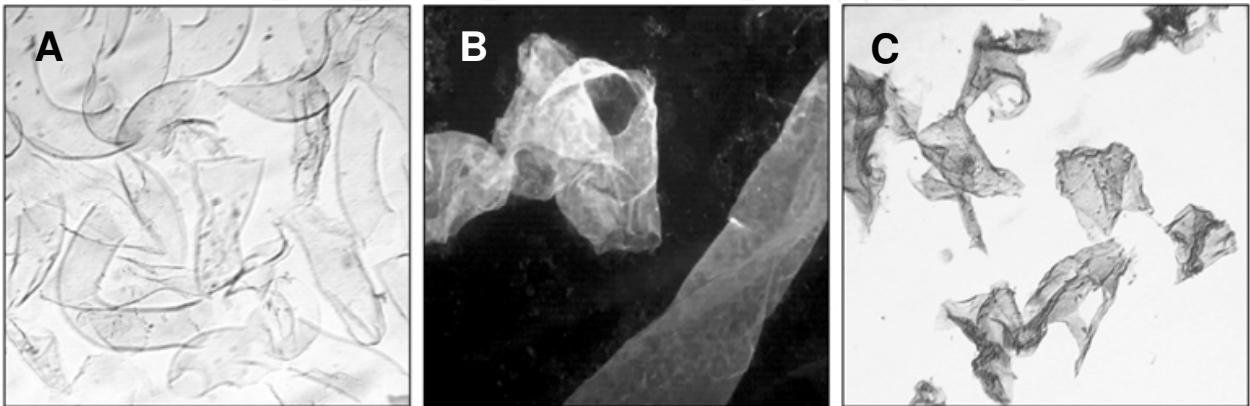


Fig. 9. Seminiferous tubule-based particles. (A) Fragments of isolated seminiferous tubules. (B) Immunostaining of isolated seminiferous tubules, showing the retention of laminin during the processing. (C) Seminiferous tubules particles after further fragmentation of isolated tubules

3.2 Cell compatibility of beads

To be useful, any bead must clearly not be cytotoxic and must allow ready attachment and growth of cells. All the beads and particles described above (Figs. 3-9) meet these criteria. There was no evidence of any cytotoxicity due to residual glutaraldehyde that had been used to crosslink some of the beads.

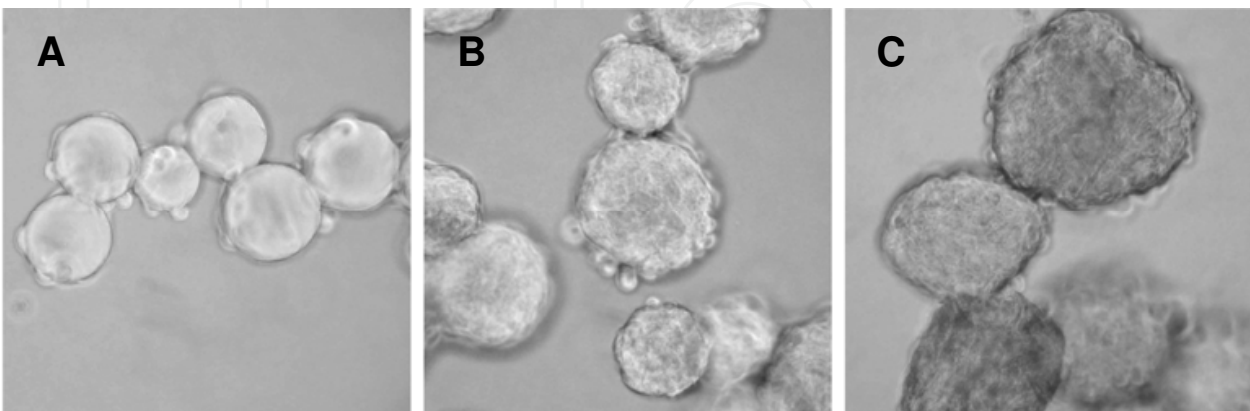


Fig. 10. Gelatin-based beads with chondrocytes after 3 days in spinner culture, showing good attachment and retention of the round chondrogenic phenotype. (A) Gelatin beads, (B) Gelatin/Type I collagen beads, (C) Gelatin/Type II collagen beads

As an example, chondrocytes readily attach to gelatin and gelatin/collagen beads and are viable and starting to proliferate after 3 days in culture (Fig. 10). Similarly, cells readily attach and start to proliferate on bone (Fig. 11A) and demineralised bone particles (Fig. 11B). Attachment and growth of chondrocytes was good on the seminiferous tubule particles, even after only 2 days in culture (Fig. 12).

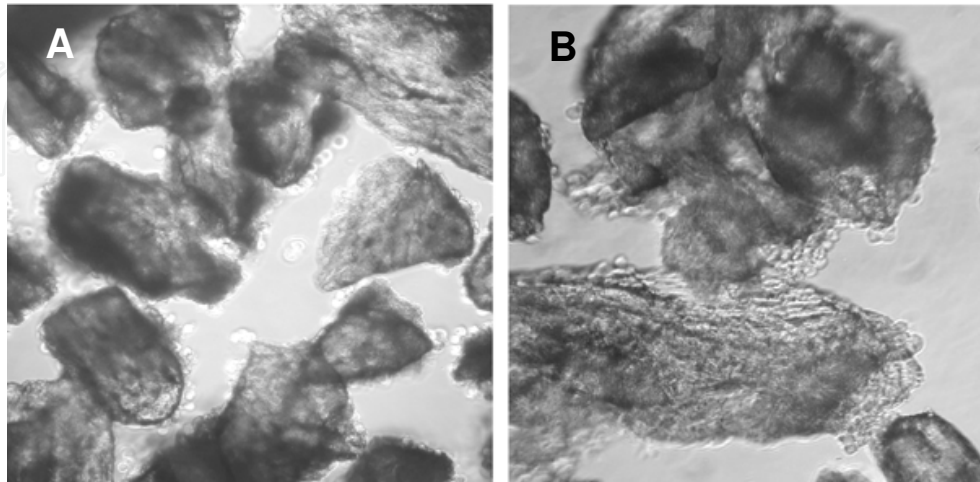


Fig. 11. Chondrocytes growing on bone particles. (A) Sheep chondrocytes on bone particles after 3 days in spinner culture, (B) Human chondrocytes on demineralised bone particles after 4 days in spinner culture

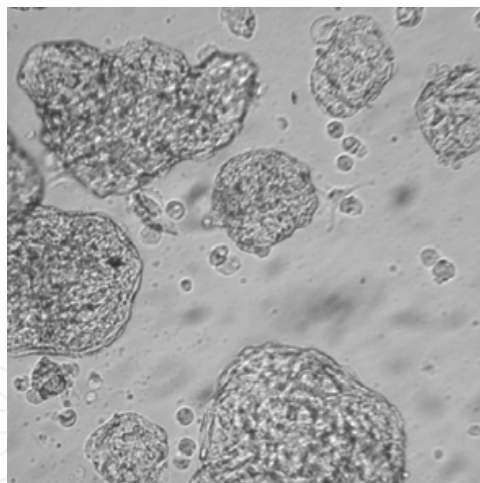


Fig. 12. Human chondrocytes growing on bovine seminiferous tubule particles for 2 days

3.3 Cell growth rate on beads

Previous studies (Fronzoza et al., 1996) had suggested that cell proliferation of chondrocytes on synthetic microcarrier beads was superior to that found in monolayer culture. The present work using biologically based beads supports this finding (Fig. 13). Thus, over a 21 day period, with cell collection and re-seeding after 7 and 14 days, the spinner culture using both gelatin and demineralised bone particles was significantly better than that found using monolayer cultures. In all cases, the bead experiments were established so that the surface area of the carriers was equivalent to that used in the monolayer cultures. The cell yield for

the gelatin beads was almost one order of magnitude greater than with monolayer cell culture (Fig. 13). The performance of demineralised bone particles was even better, being better than 2 orders of magnitude greater than for monolayer culture. As similar culture conditions were used for both the gelatin and demineralised bone particles, this reflects the differences in surface compositions and the surface topography of the two bead types as well as the potential for the demineralised bone particles to contain some active growth factors that may boost cell proliferation (Wildemann et al., 2007). Comparisons in an additional experiment including other bead systems (Table 1) showed that proliferation rates were consistently better for biological, collagen-based beads when compared to monolayer culture and a synthetic bead.

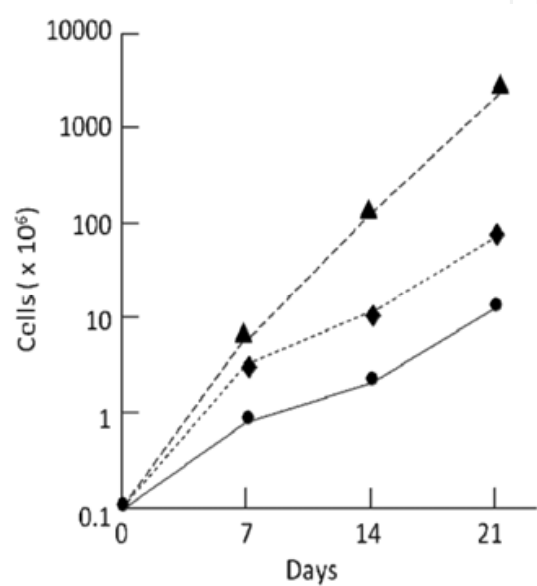


Fig. 13. Proliferation of human chondrocytes in monolayer culture and in spinner culture using different microcarrier systems. The initial loading was 0.25×10^6 cells on beads that gave equivalent surface areas of $\sim 125 \text{ cm}^2$ (equivalent to 5 T25 monolayer flasks). ● monolayer culture, ◆ spinner culture using gelatin beads, ▲ spinner culture using demineralised bone particles

Culture system	Bead/particle type	Proliferation rate after 21 days
Monolayer	n/a	5.5
Spinner Culture	Cytodex	6.5
Spinner Culture	Gelatin	38.4
Spinner Culture	Bone	37.2
Spinner Culture	Demineralised bone	47.2

Table 1. Comparison of the proliferation rate for human chondrocytes obtained using different culture systems. The proliferation rate was determined relative to the initial number of cells that were seeded

3.4 Extracellular matrix production in culture

In the present study we have focussed principally on chondrocytes. In these cases, with a variety of different beads in spinner culture, newly formed extracellular matrix was found

to accumulate around the beads and cells, causing clumps of tissue-like material to form in the cultures. This was clearly evident by 7 days, for example, with gelatin and gelatin/collagen beads (Fig. 14), where a matrix of cells and collagen can be seen holding most beads in clumps.

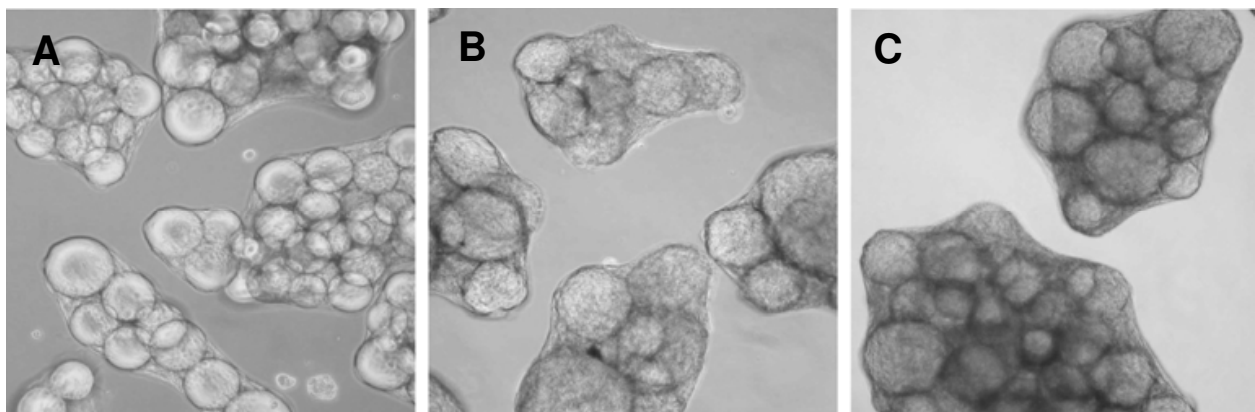


Fig. 14. Growth of chondrocytes on various beads after 7 days in spinner culture, showing the clumping of beads due to the formation of new extracellular matrix. (A) Gelatin beads, (B) Gelatin/Type I collagen beads, (C) Gelatin/Type II collagen beads

Histology, using Haematoxylin and Eosin staining (Fig. 15) shows the presence of this new connective tissue with cells throughout. Not all cells have migrated from the beads into this new matrix and some are still stained on the bead surfaces. A similar result is seen for chondrocytes grown in spinner culture on bone particles (Fig. 15C).

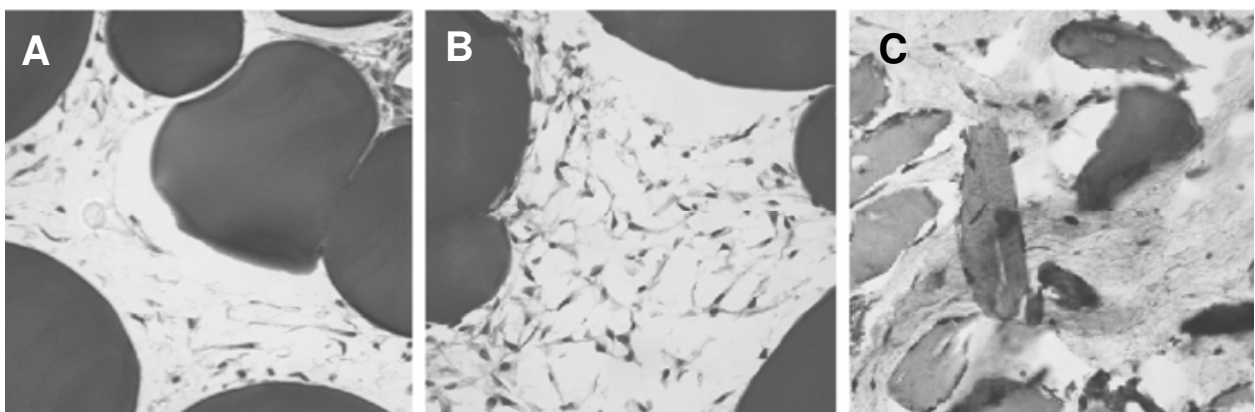


Fig. 15. Haematoxylin and Eosin staining of the growth of chondrocytes on various beads after 7 days in spinner culture, showing the new extracellular matrix that is formed (A) Gelatin beads, (B) Gelatin/Type I collagen beads, (C) Bone particles

With the porous collagen beads, the initial phase of cell proliferation was mainly infiltration of the collagen bead and less accumulation of cells and extracellular matrix external to the particles and hence less clumping was observed than with the gelatin beads (Fig. 16). On the other hand, a small amount of clumping of particles was also observed when human mesenchymal stem cells were grown on decalcified endosteal particles for 7 days. Cell attachment was good and proliferation had commenced, but was not as advanced as seen for chondrocytes on other bead systems (Fig. 17).

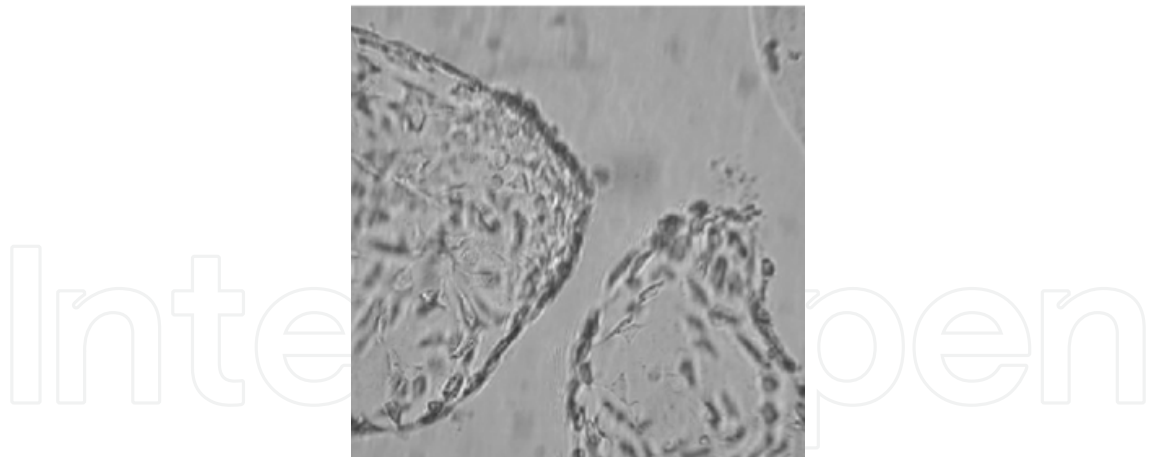


Fig. 16. Growth of chondrocytes for 7 days on porous collagen beads prepared through alginate removal (Tebb et al., 2007), showing infiltration of the beads by cells

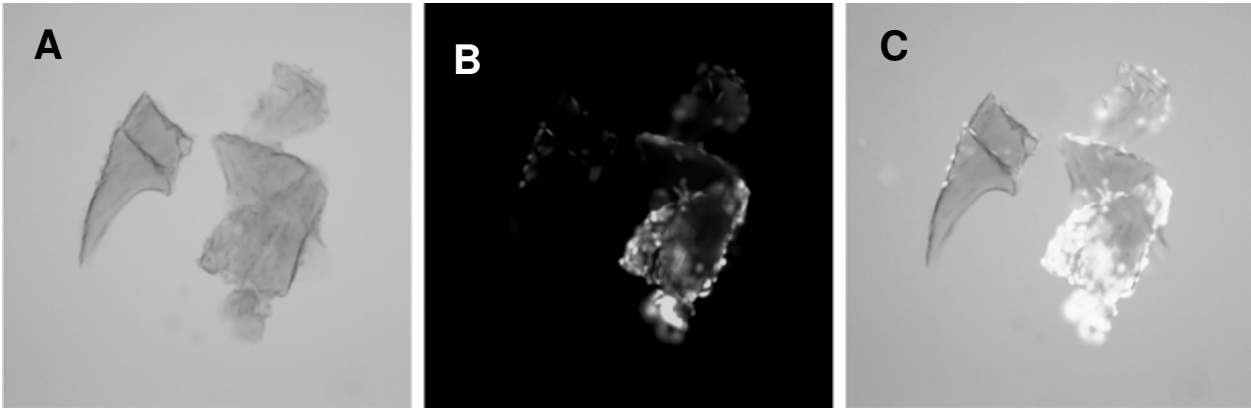


Fig. 17. Growth of human MSCs for 7 days on decalcified endosteal particles. (A) Cells attached to particles, (B) staining with Calcein-AM, which stains cell cytoplasm, and (C) Merging of the previous 2 images

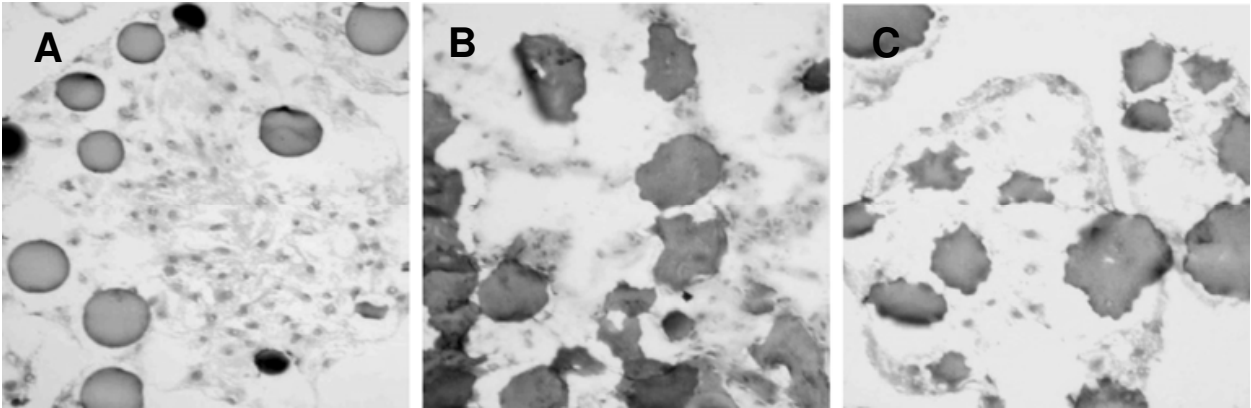


Fig. 18. Haematoxylin and Eosin staining of the growth of chondrocytes on various beads after 21 days in spinner culture, showing the formation of new extracellular matrix. (A) Gelatin beads, (B) Gelatin/Type I collagen beads, (C) Gelatin/Type II collagen beads

After longer periods of culture, 14-21 days, continued extracellular matrix production was seen when ascorbate was included, and cell proliferation also occurred. After 21 days,

Haematoxylin and Eosin staining of materials containing gelatin and gelatin/collagen beads showed extensive matrix between particles (Fig. 18), which were now more separated than in the 7 day clusters (Figs. 14 & 15). In addition, the particles containing collagens were now showing erosion and degradation (Fig. 18B, Fig. 18C), while the gelatin alone particles were still substantially intact (Fig. 18A). Alcian Blue staining of these samples confirmed that the gelatin alone beads were more stable than those containing collagen (Fig. 19) and showed that the extracellular matrix contained proteoglycans/glycosaminoglycans which are key products of chondrocytes and are indicators of proper cell function and cartilage formation. The staining was strongest around the periphery of the particles where cells were still attached. The chondrocyte cell phenotype was also examined by immunohistology (Fig. 20). This showed that after 14 days in spinner culture the chondrocytes were still producing type II collagen, the correct collagen for articular (hyaline) cartilage formation, and the amount of type I collagen being produced was very low (Fig. 20).

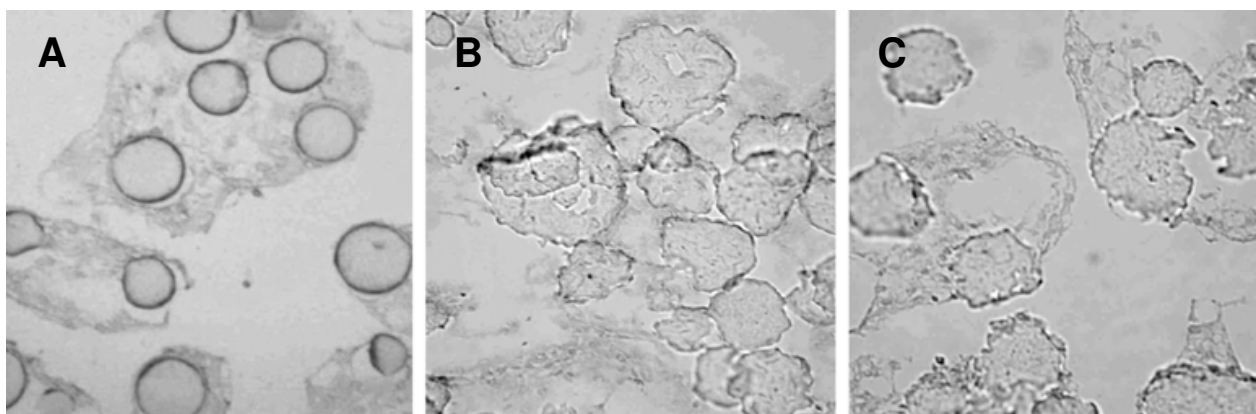


Fig. 19. Alcian Blue staining of the growth of chondrocytes on various beads after 21 days in spinner culture, showing the formation of proteoglycans within the new extracellular matrix. (A) Gelatin beads, (B) Gelatin/Type I collagen beads, (C) Gelatin/Type II collagen beads

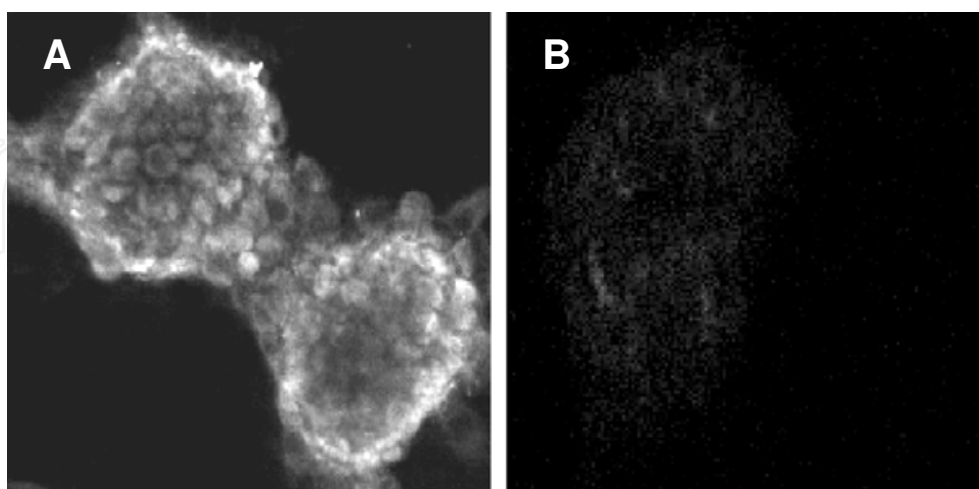


Fig. 20. Immunohistological staining of chondrocytes grown on gelatin beads in spinner culture for 14 days, showing continued production of type II collagen and little, if any type I collagen production. (A) Immunostaining for type II collagen, (B) Immunostaining for type I collagen

3.5 Bead culture in a collagen carrier gel

It was noted earlier, in the Introduction, that if cells are expanded for cell therapy on biodegradable, resorbable beads, then there is no need to isolate the cells from the beads prior to administration (Glattauer et al., 2010; Werkmeister et al., 2006), and that this has the advantage of minimizing the extent of cell handling that is required. In such a system, delivery of the cells/bead in a gel or gel forming material has further advantages. The presence of the gel stabilises the cell implant, and aids in the delivery of the cell/bead constructs.

Collagen type I gel is an ideal FDA approved protein that can be used as the delivery vehicle. When mixed with the cell/bead constructs the mixture can still be readily delivered through a 22-gauge needle, while the particles with bound cells remain uniformly dispersed and do not settle out rapidly (see Fig. 21A, where the particles have been stained to highlight the distribution in the gel). On warming to 37 °C for 1 h, the collagen formed a fibrous, solid gel that was able to be picked up, if required (Fig. 21B). This would not be necessary for in situ application of the mixture by injection into tissue. Indeed, we have injected a solution of cell/bead constructs in collagen gels and found that the mixture gelled in situ and gel contraction was markedly reduced by the presence of the beads (data not shown).

The cell/bead constructs embedded in a collagen gel can be maintained in culture for greater than 3 weeks. During the initial stages of culture, within 3 days, if synthetic, unmodified Cytodex™ beads are used, the cells show a greater affinity for the collagen of the gel than the bead and migrate from the beads into the surrounding gel. Cell migration is much slower from gelatin and other biologically-seeded beads where there seems a possible better affinity for the bead.

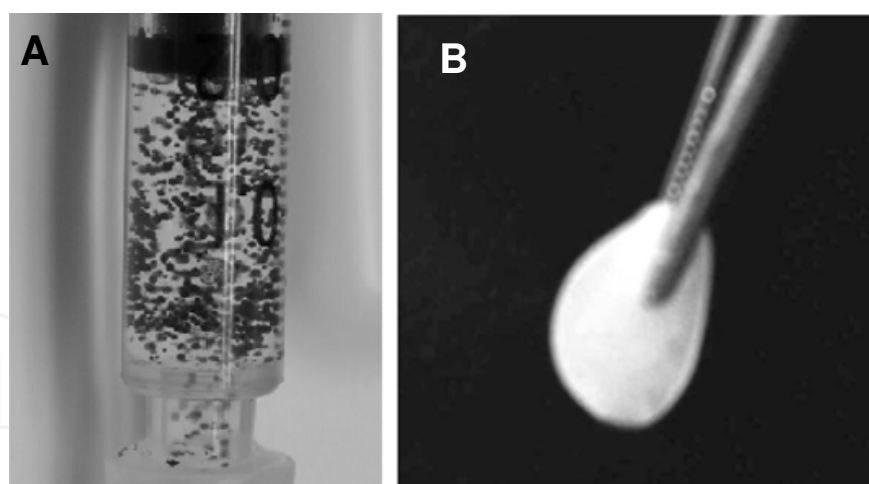


Fig. 21. Delivery of cell/bead constructs using a collagen gel. (A) Stable dispersion of gelatin beads, after staining, in a 1% collagen type I gel. (B) Collagen gel with beads, after heating to 37 °C forms a mechanically stable gel

After 3 weeks in static culture, the cell/gelatin beads/collagen gel construct had a tissue-like appearance (Fig. 22), and the loose structure of the original gel construct had formed a firmer, more compact structure, which showed very minimal contraction. When cells are seeded into collagen gels alone, the gel contracts significantly, by ~50% or more, within the first 7 days of culture (Ramshaw et al., 1991) and again forms a more compact tissue-like format.

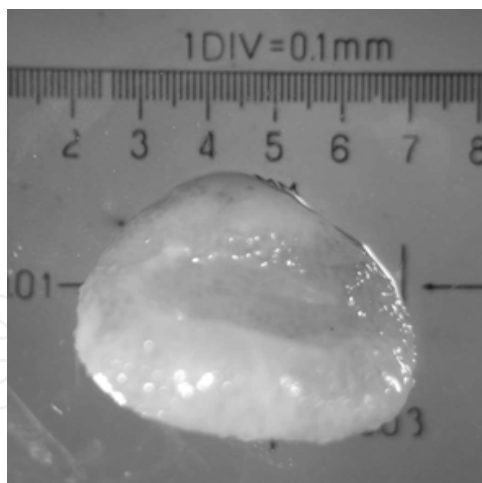


Fig. 22. A collagen type I gel with cells/gelatin beads after 3 weeks in static culture, showing the tissue-like material that is formed

The presence of the beads appears to retard any contraction of the collagen gel, while at the same time the synthesis of extracellular matrix augments the structure. Chondrocytes in culture can produce matrix metalloproteinases (Lefebvre et al., 1990) which could degrade the construct. The presence of the beads, where the cells have formed an established matrix prior to incorporation in the collagen gel may minimise the production of these proteinases. However, after 2 to 3 weeks of culture some erosion of gelatin beads was seen (Fig. 23A, Fig. 23B). The extent of this erosion was greater than when the collagen delivery gel was absent (Fig. 18A).

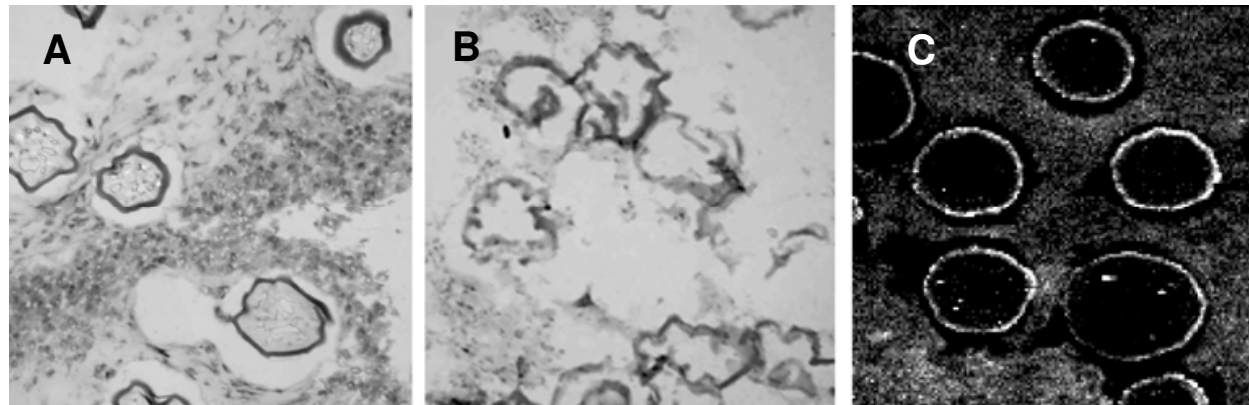


Fig. 23. Histological examination of cell-bead constructs in a collagen gel after 2-3 weeks in culture. (A) Haematoxylin and eosin staining after 2 weeks of culture, (B) Alcian Blue staining after 3 weeks in culture, and (C) Immunostaining of Type II collagen after 2 weeks in culture

The presence of new extracellular matrix can be seen by histochemical and specific immunohistological staining. Heamatoxylin and Eosin staining after 2 weeks of culture (Fig. 23A) shows extensive cell infiltration of the collagen gel, and new collagen synthesis around the beads and within the gel. Alcian Blue staining after 3 weeks of culture (Fig. 23B) shows significant deposition of proteoglycan/glycosaminoglycan within the gel surrounding the beads, as well as very strong staining around the beads themselves. This staining was not present in the original collagen gel and is therefore indicative of new matrix production.

This is also the case for type II collagen production. Immunohistology after 2 weeks of culture (Fig. 23C) shows that there is type II collagen accumulation, possibly at low levels, throughout the matrix, while there is strong staining around the beads.

4. Conclusions

The data presented in this Chapter show that a wide variety of collagen-based beads and particles can be made which are useful for spinner culture. The beads can be made with a range of tissue-like conformations, ranging from denatured collagen (gelatin) through to beads that are based around basement membrane and collagen type IV structures. The beads, including those that have been further crosslinked using glutaraldehyde, were not cytotoxic and readily supported cell growth. The cell growth in spinner culture was excellent and within 7 days there was normally accumulation of new extracellular matrix. For cell therapy applications, the proliferation rates of chondrocytes were excellent, several fold better than monolayer cultures and synthetic beads. While most biological tissue based particles or biologically fabricated beads were excellent cell carriers, demineralised bone particles and gelatin beads were particularly good, the former possibly augmented by residual growth factors. In a therapeutic application the demineralised bone-based beads could have a fairly long resorption rate. If a shorter resorption rate was preferred, then gelatin beads are ideal, and the rate of resorption can be controlled by varying the extent of glutaraldehyde stabilisation (Glattauer et al., 2010). After longer times in culture, exceeding 21 days, the chondrocytes maintained their phenotype and were still producing macromolecules characteristic of native articular cartilage, such as proteoglycans and type II collagen. For therapeutic applications a superior approach is to deliver the cell/bead constructs in a delivery gel; in the present study a gel of type I collagen has been used. In this case the choice and design of the beads can be selected to match the requirements with respect to cell type and bead resorption rate. These gel constructs were shown to be stable, retaining their size and becoming more robust with new matrix accumulation. Again the chondrocytes maintained their phenotype over 21 days, as shown by proteoglycan and collagen type II production.

Together, these data show the utility of expanding cells on biological, collagen-based beads in spinner culture for tissue engineering and cell therapy applications. Further, these data also show that an ideal approach is to use resorbable beads for cell expansion and then direct delivery of cells still attached to the beads, where it is possible to design carrier beads with a range of stabilities that matches the implant requirements. An ideal way of achieving this implant is to combine the cells/beads together with a delivery gel. This proposed approach has been used in an animal trial for articular cartilage repair, where it was compared to a standard ACI approach (Chiang et al., 2005). The results strongly confirmed the validity of the proposed method, with an independent multifactor scoring system showing that the cell/bead/gel approach was significantly better than a standard ACI procedure and a non-cell control in a mini-pig model (Chiang et al., 2005). The approach can be further improved by examining delivery systems that allow the delivery and stabilisation of the cell/bead construct by arthroscopy, removing the need for open knee surgery. Equally, although the present techniques use cartilage biopsies obtained from non-load bearing cartilage, recent developments in cell biology, for example the ready acquisition of mesenchymal stem cells which can be differentiated into chondrocytes, opens up further opportunities that may minimise invasive surgery for the patient.

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Regenerative Medicine and Tissue Engineering - Cells and Biomaterials

Edited by Prof. Daniel Eberli

ISBN 978-953-307-663-8

Hard cover, 588 pages

Publisher InTech

Published online 29, August, 2011

Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

How to reference

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Veronica Glattauer, Wei-Bor Tsai, Jacinta F. White, Julie Nigro, Tracy A. Tebb, Jerome A. Werkmeister and John A.M. Ramshaw (2011). Direct Use of Resorbable Collagen-Based Beads for Cell Delivery in Tissue Engineering and Cell Therapy Applications, *Regenerative Medicine and Tissue Engineering - Cells and Biomaterials*, Prof. Daniel Eberli (Ed.), ISBN: 978-953-307-663-8, InTech, Available from: <http://www.intechopen.com/books/regenerative-medicine-and-tissue-engineering-cells-and-biomaterials/direct-use-of-resorbable-collagen-based-beads-for-cell-delivery-in-tissue-engineering-and-cell-thera>

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