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Metabolic and histological analysis of mesenchymal stem cells grown in 3-D hyaluronan-based scaffolds

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Sheep mesenchymal stem cells (MSCs) were isolated and expanded using the principle of plastic adherence. Their identity as progenitor cells was confirmed by induction along the osteoblastic lineage using osteogenic supplements and observation of calcific deposits by von Kossa staining. MSCs were seeded onto two types of hyaluronan-based cylindrical scaffolds in high concentrations and cultured for varying time points up to three weeks. Culture medium was supplied using the following conditions: statically, on a shaker, by stirring with a magnetic stirrer or by perfusion in a tubular flow circuit. Total cell metabolism was assessed by MTT assay and the quality of cell coverage and matrix formation observed by SEM and histological analysis of thin sections of the constructs. Perfusion culture was established as the most appropriate culturing conditions, with cell metabolism increasing by approximately 300% over three weeks. The coverage of the scaffold surface was very good and the deposition of collagenous matrix was superior in these conditions compared to the static and other dynamic culture conditions.

Introduction

The discipline of tissue engineering requires the creation of *de novo* tissue using cells that are generally seeded onto a porous scaffold [1]. The specifications for the physical characteristics of the scaffolds, including interconnecting minimum pore size, etc., have often been described using empirical studies rather than a robust engineering analysis. However, beyond certain magnitudes of dimensions, even these empirical specifications can be insufficient to sustain the nutritional requirements of the seeded cells in static culture. During the phases of cell proliferation and expression of extracellular matrix within the scaffold, the barriers to satisfactory nutrition become even greater. However, for specific geometries, the required methods of dynamic culture medium delivery are ill defined, even though this early phase of tissue development may be of critical importance to the final product.

Studies of construct development in dynamic systems often assess cell number (e.g. proliferation rate), although the manner of matrix expression, it's composition and the orientation of cells could be more important. In the study described in this communication, we wished to create a viable analogue of a ligament such as the anterior cruciate ligament (ACL), and our attempts to optimise conditions were assessed.

Using ovine mesenchymal stem cells (MSCs) as the cell source, two hyaluronan-based cylindrical tissue engineering scaffolds with different internal fibre geometries were seeded with cells, cultured for varying periods up to three weeks in static and various dynamic culturing arrangements and then compared for cell metabolism, cell and matrix surface coverage and internal structure using SEM and conventional histology. The health of the cells within the scaffolds was monitored using a vital stain and confocal microscopy.

Materials and methods

Bone marrow was extracted from sheep (Merino, South prealp or Cambridge-cross) immediately after either lethal injection or stable anaesthesia with

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O_2–N_2O–halothane from the iliac crest using seven-gauge trephine biopsy needles (Rocket Medical, Watford, UK) and placed immediately into 3000 IU heparin. The samples were cooled on ice prior to isolation during transportation. The procedure for MSC isolation was largely that of Solchaga [2]. The marrow was washed in DMEM (Invitrogen, Paisley, UK) with 10% fetal calf serum (FCS), particulate debris removed and any cell aggregates dispersed by forced passage through a 21-gauge needle. The marrow was plated-out at a concentration of $3.6 \times 10^5/\text{cm}^2$ in T-75 culture flasks and maintained at 37 °C in 5% CO_2. At each stage of MSC isolation and expansion cell numbers were measured for each sample. Medium was changed subsequently twice per week with DMEM with 10% FCS. The FCS had been batch tested against other supplies to determine the serum which produced the most desirable degree of proliferation on identical cell populations. To allow quantitative data to be compared, a large quantity of the best batch (Australian origin from Invitrogen, Paisley, UK) was purchased, frozen and stored until required.

The cells were passaged just prior to confluency by detachment using 0.25% trypsin in EDTA. The identity of the cells as MSCs was verified by testing the ability of cell colonies to express both osteoblastic and chondrocytic functionality after culture with osteogenic and chondrogenic supplements, respectively [2, 3]. In each case, each supplemented sample was compared with cells from the same sample but without the differentiating supplement. The osteogenic supplement comprised 100 μM ascorbate-2 phosphate, 10^{-7} M dexamethasone and 10 mM β-glycerophosphate as final concentrations, all purchased from Sigma (Poole, UK). The chondrogenic medium was DMEM-HG (high glucose) supplemented with 1% ITS + Premix (standard supplement containing insulin, transferrin and selenous acid, purchased from BD Biosciences, Oxford, UK), 100 μM ascorbate-2 phosphate, 10^{-7} M dexamethasone and 10 ng/ml TGF-β1 (all three from Sigma Poole, UK). Chondrogenesis was achieved by spinning 2.5×10^5 MSCs at 1000 g for 5 min in a 15 ml polypropylene conical tube (Falcon, Oxford, UK) and culturing the resultant pellet in the chondrogenic medium at 37 °C in 5% CO_2. Medium was changed twice a week for the osteogenic samples, and every other day for the chondrogenic samples. To test for osteogenesis, cultures and controls were stained with von Kossa after three weeks in culture. The chondrogenic samples were fixed in 10% neutral buffered formalin, embedded in paraffin, then 6-μm serial sections of the block were stained with Toluidine Blue.

Many different methods for seeding the scaffolds were employed, including different cell concentrations, cell numbers, and strategies for air nuclei removal and homogenous internal cell infiltration. Finally, it was determined that cells at a concentration of $7.5 \times 10^7/\text{ml}$ in DMEM be utilised, with 7.5×10^6 cells per cm linear length of scaffold required for seeding. This was performed by layering the required volume of MSCs onto the exterior of the scaffold in an equal distribution following removal of air bubbles by applying vacuum to scaffolds pre-incubated in FCS and excess serum removal using a pipette.

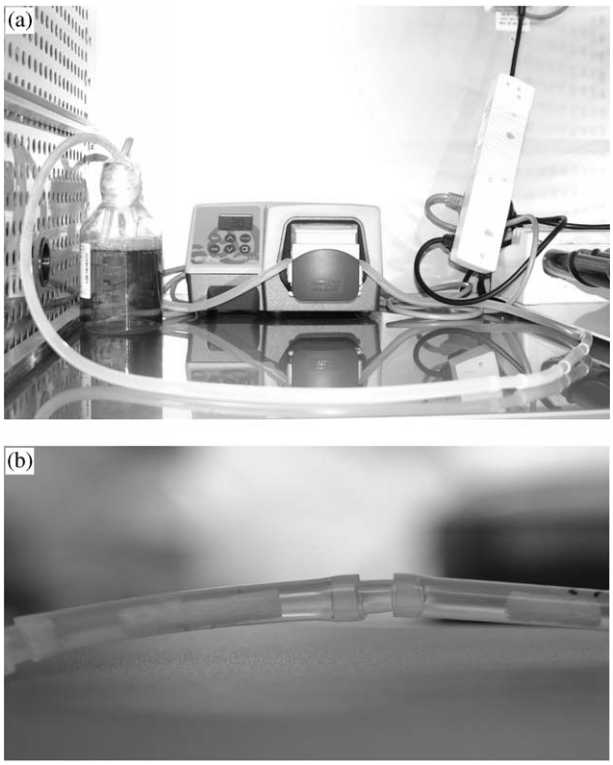

Figure 1 Tubular perfusion circuit used for active cell nutrition in the HYAFF scaffolds.

When sufficient MSCs had been cultured, 3 cm lengths of the scaffolds were seeded with the ovine MSCs. The constructs were incubated either statically, or dynamically by shaking on an orbital shaker, mixing with a stir bar on a magnetic stirrer or by perfusion by placement of the construct in 10 mm internal diameter silicone tubing and continuous flow of culture medium over the construct by peristaltic pump at a flow rate of 15 ml/min (Fig. 1). After 3, 7, 14 and 21 days, the constructs were removed from culture and bisected, with one half treated with MTT and the resultant tetrazolium conversion measured colorimetrically. The remaining half of each scaffold was embedded in glycol methacrylate (GMA resin), sectioned and examined histologically using van Geisson, to indicate collagen formation, and haematoxylin and eosin for cell location and morphology. Some pieces of scaffold were also retained for examination under scanning electron microscope (LEO 1550, Cambridge, UK) after sputter coating with chromium. In some samples, the status of the cells within the scaffold were assessed by staining with calcein AM and ethidium bromide, a live–dead assay which stains the live cells green and dead cells red. Scaffolds were carefully sectioned after staining and the samples viewed using laser-scanning confocal microscopy (Zeiss LSM-510).

The scaffolds were cylindrical, of approximately 1 cm diameter and constructed solely from fibres of esterified hyaluronan (HYAFF-11) from Fidia Advanced Bioploymers (Abano Terme, Italy). Two versions of the scaffolds were used (T14 and T18), that varied only in the geometry and orientation of the internal fibres.

Scaffolds were sterilised with γ-radiation prior to cell culturing.

Results

The total cell metabolism within the two scaffold types increased in all culture conditions over time. But whilst there was an increase of approximately 50% in MTT conversion in the static culture, there was a three-fold increase in cell metabolism in the shaking and perfusion cultures (Fig. 2). The differences in cell metabolism in the different scaffolds (T14 and T18) were not significantly different, but the differences between static or stirring culture and perfusion or shaking culture were highly significant ($p < 0.05$), $n = 5$. It was possible to view compromised cells within the interior of any of the scaffolds, but the perfused samples had the most live cells (Fig. 3). Indeed, the effort put into ensuring homogenous seeding was vindicated by the images obtained using this technique.

The visible external surfaces of the samples also varied considerably. Perfusion consistently gave a better coverage of HYAFF fibres than did the other culturing methods (Fig. 4). There appeared to be complete confluence of the cells using this technique. This was confirmed by histological analysis (Fig. 5), where the collagenous content of the constructs was thicker, but also there was a greater degree of apparent organisation in the extracellular matrix following culture in perfusion conditions.

For other culture medium delivery systems (static, stirring and shaking), although cell metabolism was not necessarily impaired, maturation of the cultures was not observed, MSCs not being particularly well oriented or producing much matrix, indicating that little differentiation was occurring in these culture conditions.

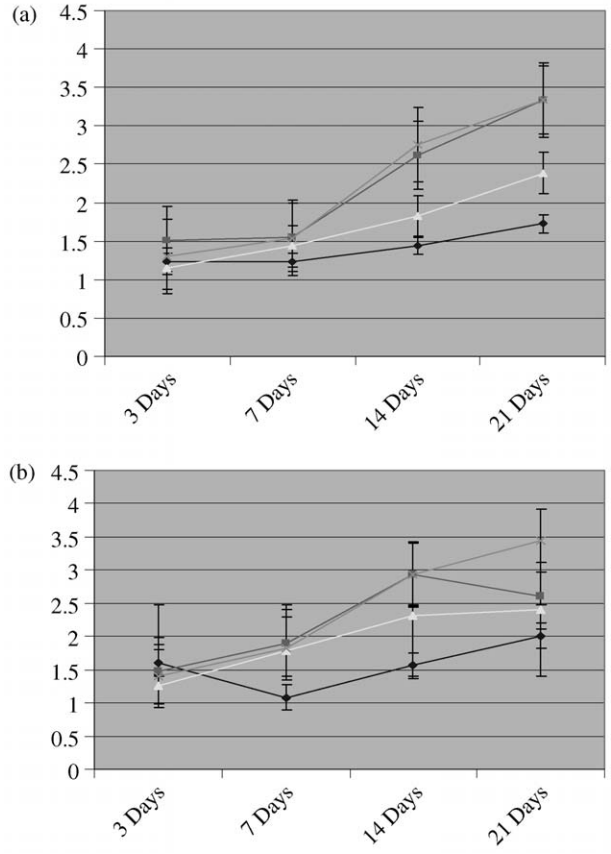

Figure 2 Cell metabolism (MTT assay) over 3 weeks for MSCs grown in different culturing conditions for (a) T14 scaffold; (b) T18 scaffold, $n = 5$, mean ± SD; white: stirring; dark grey: shaking; black: static; light grey: perfusion.

Discussion

Although proliferation of stem cells in 2-D culture is well established [4, 5], the creation of a highly organised 3-D, metabolically active cell populations is a real challenge. As tissue maturation proceeds, the provision of a nutritionally viable environment becomes even more challenging. Many researchers in the field of tissue engineering now propose bioreactors [6] that provide a sterile environment but with sufficient delivery of culture medium to prevent necrosis, but often generic solutions provide little difference from the stirring conditions described in this study.

For different tissue engineering applications, the requirements of cellular organisation and extracellular matrix deposition are specific to the application. For tendons or ligaments, for example, it is necessary to encourage the formation of fibroblastic phenotypes and the production of large quantities of collagenous matrix. The static cultures clearly appear to have not resulted in cell deposition that is very organised, and little evidence of matrix production. Cells appear to possess a fairly round morphology, suggesting that prolonged culture on the HYAFF scaffold has not resulted in the cells being directed along any particular lineage. The results of this study do, however, confirm that HYAFF-11 is a suitable material for supporting the growth of MSCs and that under the correct conditions these cells can be encouraged to grow into the nutritionally-challenging tight interstices of a fibrous scaffold whilst producing collagenous extracellular matrix.

Indeed, the perfused scaffolds contained cells exuding collagenous matrix both internal and external to the scaffold. The outer layer of these scaffolds had a thick carpet of cells and matrix as demonstrated by histology and SEM. This suggests that the cells were more fibroblastic in their phenotype, and certainly not chondrocytic, osteoblastic or adipocytic in their nature, the other lineages along which it is possible for MSCs to differentiate. It was also clear, however, that this matrix was expressed in-between the fibres.

There is much debate about the need to provide biomechanical stimulation to progenitor cells in tissue engineering constructs in order to provide the correct cues to kick start cell differentiation. Many tissue engineering solutions work better when the cell-seeded scaffolds are implanted at an early stage, the physiological environment providing the necessary biochemical and biomechanical signalling. Clearly, this may not be an option for tensile structures such as ligaments. In the case of the perfusion culture, however, some differentiation

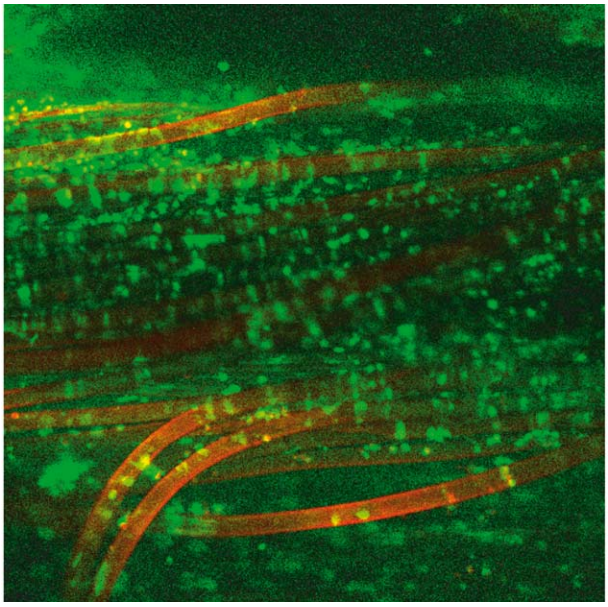

Figure 3 Live – dead assay (calcein AM/ethidium bromide) of MSCs in a HYAFF-11 scaffold after three weeks in perfusion culture; green: live cells; red: dead cells and HYAFF fibres.

appears to be occurring and it is proposed that the mechanical pressure of the flowing culture medium may be sufficient to provide the necessary signals.

A further consideration in the decision about the timing of the implantation is the protection that may be afforded to the cells within the construct when surrounded by a thick layer of extracellular matrix. It is sometimes proposed that tissue engineering solutions are likely to fail merely due to the invasion of the host inflammatory response, recruited by the surgery itself. In the case of the constructs developed within the perfusion culture arrangement, it is clear that such a construct would be much more robust and less susceptible to phagacytosis than an undeveloped analogue implanted directly following cell-seeding.

Conclusions

Cell metabolism in a 3-D, MSC-seeded scaffold was shown to be compromised in static and stirring culture compared to shaking and perfusion cultures. Additionally, cell orientation and matrix production in all the non-perfused conditions was poorly developed with little evidence of cell differentiation. In the perfused cultures, however, thick layers of extracellular matrix were observed both in the internal spaces and on the exterior of the scaffolds, with cell that appeared to be more fibrobastic. It is proposed that in addition to satisfactory nutritional support, the perfusion environ-

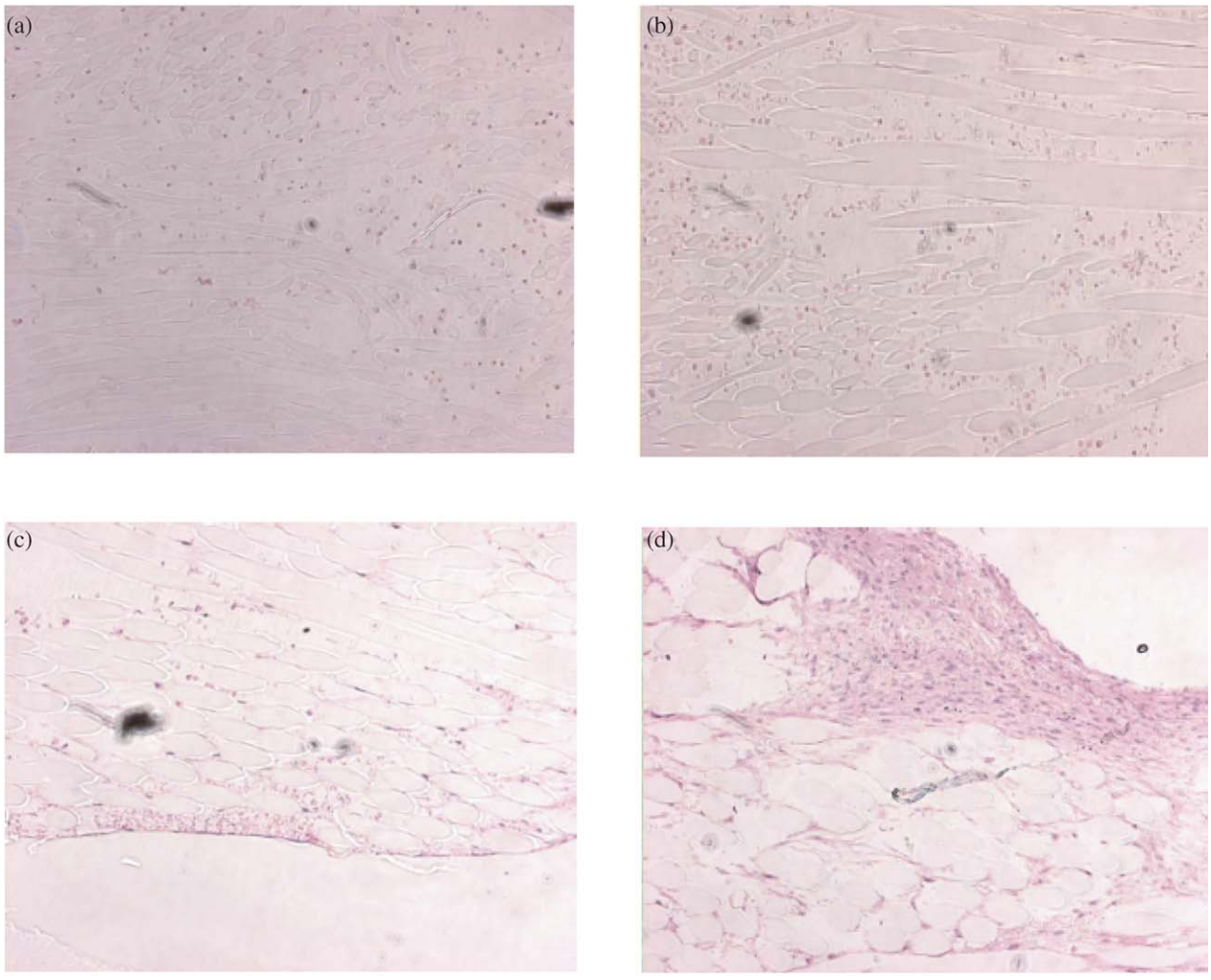

Figure 5 Histology of HYAFF scaffolds after culture for two weeks in either (a), (c) static or (b), (d) perfusion conditions. Stains are haematoxylin and eosin for (a) and (b) and Van Geisson for (c) and (d).

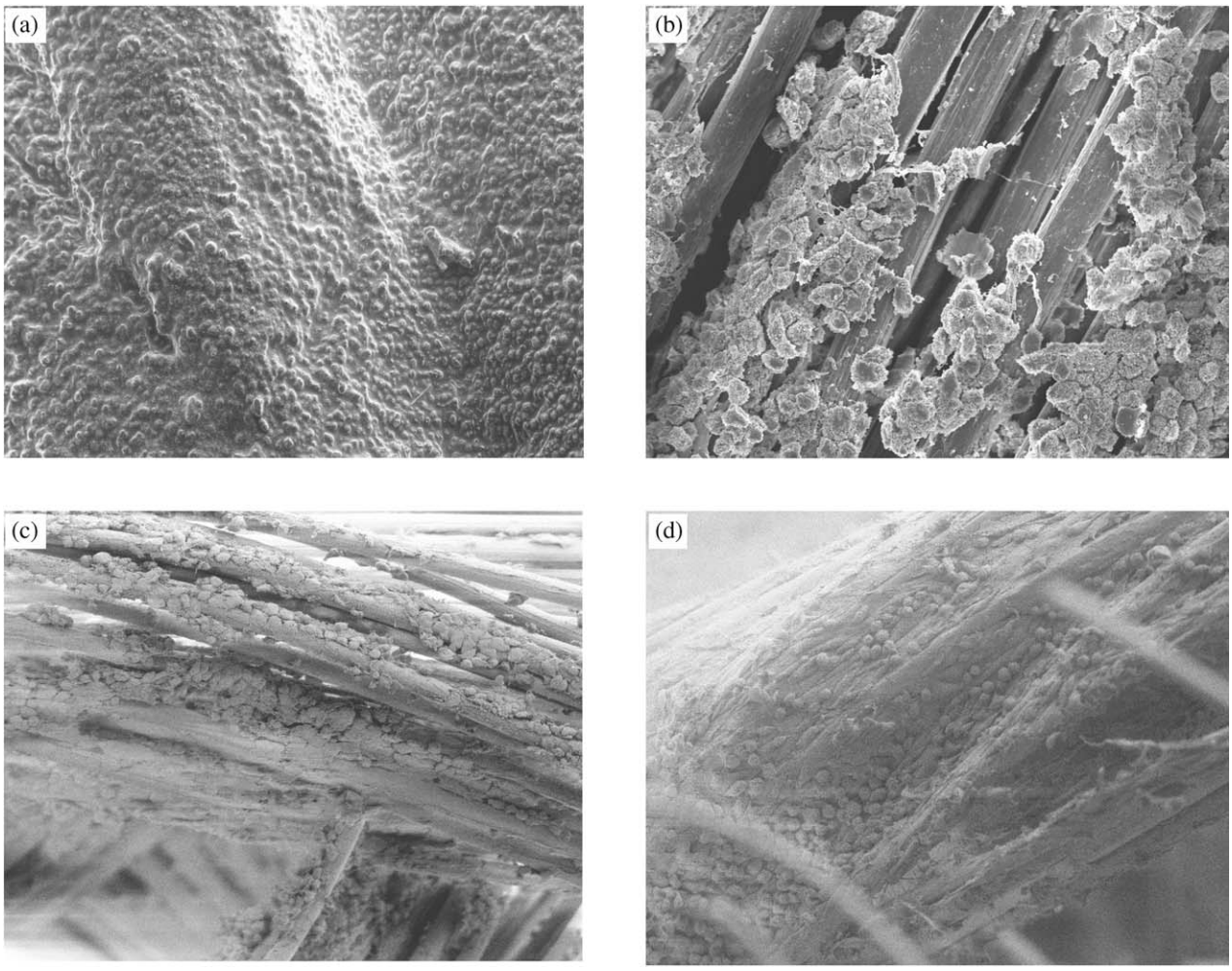

Figure 4 SEM images after three weeks in culture (a) perfusion; (b) static; (c) shaking; (d) stirring.

ment provides the necessary mechanical cues to allow tissue maturation relevant to ligament analogues.

Acknowledgments

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