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Studies on the use of hollow fibre membrane bioreactors for tissue generation by using rat bone marrow fibroblastic cells and a composite scaffold

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Abstract Production of sufficient tissue *in vitro* for use in tissue engineering is limited mainly by the absence of adequate oxygenation and appropriate transport of nutrients to, and waste product from, the tissue. To overcome the limitations of diffusive transport, the possibility of growing three dimensional (3D) tissue structures by using hollow fibre membrane bioreactors (HFMB) has been considered in this study. The hollow fibre membranes, embedded in the 3D scaffold, are porous and semi-permeable and can thus serve similar functions to arteries and veins *in vivo*. Collagen gel and Cytodex 1 microcarriers were used as a composite 3D scaffold and permeating cellulose acetate hollow fibre membranes were attached to both ends of a polycarbonate cylindrical shell to form a bioreactor. Rat bone marrow fibroblastic (RBMF) cells were seeded initially onto Cytodex 1 microcarriers and these were subsequently mixed with collagen gel before inoculation into the bioreactor. Bioreactors were perfused by culture medium through the hollow fibre membranes for a one week period. Bioreactors containing cells cultured under similar conditions except for the lack of perfusion of medium served as controls. The proliferation, viability, metabolism and morphological appearances of the cells in the perfused and non-perfused constructs were compared. The results indicated that there was significantly greater maintenance of functional activity and normal cellular morphology in the perfused group than in the non-perfused group. Further studies are required to evaluate the additional advantages of using this novel HFMB for growing 3D dense tissues.

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Introduction

New developments in the tissue engineering field are extensive with almost all tissues and organ systems under consideration. Some engineered tissues are already available commercially or under clinical trial [1]. However, currently only avascular (cartilage) or thin sheets of tissues (skin) are capable of being successfully engineered [2]. The mass of three-dimensional (3D) tissue that may be produced *in vitro*, especially dense tissue, is very limited [3]. To develop a 3D tissue, the problems of oxygen and nutrient supply to the cells and waste removal from these cells, particularly in the centre of the construct, must be addressed. Biological angiogenesis is the primary requirement for generation of an appreciable mass of most tissues, but initiation and control of angiogenesis remain a major technical challenge to tissue engineering. An engineering solution to supplying oxygen and essential nutrients to the growing tissue *in vitro,* together with removal of metabolic waste products, is the potential use of hollow fibre membrane bioreactors so that the hollow fibre membrane network mimics the blood capillary system.

The bioreactor is central to the tissue culture process and provides the pre-defined chemical, biochemical, physical and mechanical environments for the seeded scaffolds, in which cells proliferate and differentiate to form neo-tissues. Various types of bioreactors have been investigated for tissue culture, including the spinner flask bioreactor, the rotating vessel

bioreactor (slow-turning lateral vessel and high-aspect-ratio vessel), the rotating wall perfused vessel bioreactor and the perfused column bioreactor, as reviewed by Freed and Vunjak-Novakovic [4]. Also, perfused chambers have been used to encourage culture medium to reach the centre of the bioconstruct through microchannels [5]. This may function well in the initial stages when cells begin to grow and proliferate on the scaffold, but with time as the cell densities increase and extracellular matrix (ECM) accumulates, most of the microchannels may be occupied by cellular materials and finally may be occluded. In addition, especially in culture of bone tissues, any mineralization of the extracellular matrix (ECM) significantly restricts nutrient diffusion.

To overcome the diffusional limitations, we have developed hollow fibre membrane bioreactors (HFMB) with embedded hollow fibre membranes within the scaffolding constructs. The wall of the hollow fibre membranes are porous and selectively semi-permeable to medium solutes. Depending on the pore sizes, passage of small molecules (nutrients, oxygen and metabolic wastes) and macromolecules (e.g. growth factors) occurs, and cells are retained completely. The nutrients and oxygen flowing within the fibres diffuse out through the membrane are distributed more evenly within the tissue. Also, spent media and metabolic waste products permeate back into the fibres and are removed from the tissue. Therefore, a nutrient circulation system similar to the capillary system in the native tissue is created.

The concept of HFMB for use in cell culture has been investigated by numerous investigators. HFMBs have found application in monoclonal antibody production [6–8], enzymatic reactions [9, 10] and mammalian cell culture [7, 11–17]. Knazek first developed the HFMB system for cell culture in 1972 [18]. The advantages of using hollow fibre bioreactors for cell culture are noted to include: (a) the cultured cells are maintained in a more physiologically appropriate environment regarding nutrient supply, metabolic waste removal and a stable pericellular microenvironment [19]; (b) the cells can be grown to very high densities ($\geq 10^7$–10^8 cells/ml) and (c) the cultured cells are protected from major mechanical shear stresses [20]. However, using the conventional HFMB, tissue structures could not be produced because of the lack of the necessary 3D scaffolds for cell attachment and proliferation. *In vivo,* cells are supported and maintained by ECM and a similar environment is required for cells to form 3D tissue structures *in vitro*. A major difference between the design of HFMBs to be used for amplifying cell numbers and that for producing engineered tissues lies in the inclusion of biomaterial scaffolds in the latter to allow the growth of the required bulk of tissue. In the current work, we have developed novel HFMBs by including collagen gels and Cytodex 1 microcarriers as composite 3D scaffolds. Rat bone marrow fibroblastic (RBMF) cells were pre-cultured on Cytodex 1 microcarriers to allow cell attachment and proliferation and subsequently inoculated into the bioreactors with collagen gels. Similar cell populations are known to contain primitive osteogenic progenitor cells and are of potential value in skeletal tissue engineering [21]. To assess the advantages of improved perfusion on cellular development, we have used commercially available hollow fibre membranes and these formed part of the construct scaffold. In addition, perfused bioreactors were compared with non-perfused bioreactors in order to assess the advantages of improved perfusion on cellular development. The viability and proliferation of the cells accommodated within the hollow fibre bioreactors were analysed and compared after *in vitro* culture for one week. The aim of the present work was to develop a functional cell-scaffold bioreactor and to determine the advantages for culture expansion of adherent rat bone marrow fibroblastic cells.

Materials and methods

Hollow fibre membrane bioreactor (HFMB)

A simple cylindrical geometry was chosen for construction of the bioreactor. The cylinder housing (dimension 13 mm I.D. × 22 mm O.D. × 40 mm L) and side ports were fabricated in-house (Department of Biochemistry, Oxford University). The cylinder and screw caps on both ends (1, Fig. 1) were made of polycarbonate. The ports for medium (2, Fig. 1) were clinical male luer connectors and the side ports for inoculation of cells (3, Fig. 1) were clinical female luer connectors, which were glued onto the body of the cylinder.

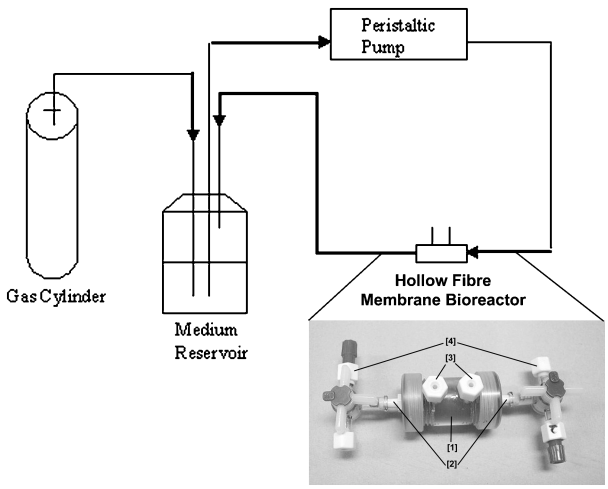

Fig. 1 Schematic drawing of the HFMB perfusion culture system. The whole culture system, except for the gas cylinder, was placed in a 37 °C incubator. 1: Cartridge (with contained hollow fibre membranes); 2: Ports for medium circulation; 3: Side ports for inoculation of cells and scaffolds; 4: Three-way stopcock, used for connection with the perfusion system and sampling

Cellulose acetate hollow fibre membranes (200 μm I.D., wall thickness of 14 μm and molecular weight cut-off (MWCO) of 10 KD) derived from haemodialyzers were used to construct the HFMBs. The hollow fibre membranes were fixed in the bioreactor by using moulded silicon rubber. The effective length of the fibre in the reactor was 30 mm with approximately 200 HFMs in each bioreactor. The distance between adjacent fibres was approximately 400 μm. The volume external to the hollow fibres in each HFMB was approximately 3.5 ml, and this volume was available for the collagen gel together with the microcarriers with adherent cells.

Cell culture

Rat bone marrow fibroblastic cells were harvested as described previously by Mardon *et al.* [22] from the femoral bone marrow of 3-month old female LOU/CN rats and pelleted by centrifugation at 300 × *g* for 5 minutes. The cell pellet was resuspended in 10 ml α-minimum essential medium (α-MEM) (Invitrogen, UK) and passed through a cell strainer (70 μm nylon, Becton Dickinson Labware, NJ, USA). Samples of cell suspension were diluted with 2% acetic acid in phosphate-buffered saline (PBS) and the number and viability of nucleated cells were determined. Cells were plated at a density of 1×10^6–5×10^6 in 25 cm^2 flasks and cultured in 20 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES) (Sigma, UK) buffered α-MEM supplemented with 10% (v/v) foetal bovine serum (FBS) (M. B. Meldrum, UK), 1% penicillin-streptomycin (Sigma, UK) and 1% Fungizone® (Gibco, UK) in 5% CO_2/95% air in a humidified incubator.

After reaching confluence, cells were recovered by treatment with trypsin-ethylenediamine tetraacetic acid (EDTA) (0.05% w/v) and then cultured with Cytodex 1 microcarriers (Pharmacia Fine Chemicals, Sweden) for 24 hours at a density of 1×10^6 cells/ml. Cell cultures on Cytodex 1 were performed following the manufacturer's protocols. In brief, 1×10^6 cells/ml were inoculated with 10 mg Cytodex/ml into a siliconised glass culture vessel. Continuous stirring was commenced once the cells had firmly attached to the surface of the microcarriers.

Cell seeding

To prepare the collagen gel solution, 2 parts distilled water and 1 part concentrated α-MEM were mixed and 5 parts of the collagen solution (3 mg/ml, supplied by Bone Research Lab, Nuffield Orthopaedic Centre, Oxford) added with gentle swirling. After adjusting the pH of the solution to approximately 7.2–7.4 using sodium hydroxide (0.4 M), 2 parts of the cell suspension (see cell culture on Cytodex 1) was added and the constituents thoroughly mixed.

The mixture was slowly injected into the extracapillary space (ECS) of the HFMBs through side ports on the shell of the HFMB (3, Fig. 1). The final cell seeding number in each bioreactor was 6×10^5 cells on 0.6 ml Cytodex 1 mixed with 2.4 ml rat tail collagen. After the injection, the bioreactors were left in the incubator at 37 °C for 30 min to allow the collagen gel to set before culture medium was introduced through the hollow fibres of the perfusion culture system or added manually through a three-way stopcock (4, Fig. 1).

Tissue culture bioreactor system

The perfusion system consisted of four major components: the hollow fibre membrane bioreactor, a peristaltic pump (205CA, Watson-Marlow Limited, Cornwall, UK), the culture medium reservoir and a gas (5% CO_2/95% air) cylinder (Fig. 1). HFMB apparatus was pre-sterilized by ethylene oxide gas and assembled under sterile conditions. The whole system was connected using sterile silicon tubes and connectors. Cells, together with other scaffolding materials, were placed in the ECS of the HFMBs. Continuously-gassed culture medium (5% CO_2 in air) was withdrawn from the medium reservoir (with 150 ml of medium) by the peristaltic pump, entered the intracapillary space (ICS) and then recirculated back to the reservoir. Nutrients and oxygen diffuse through the porous wall of the membrane and are distributed within the bioreactor. Metabolic wastes within the construct diffuse into the membrane through the porous wall and flow out of the bioreactor. The medium in the reservoir was changed on day 4 and a flow rate of 14 ml/h was used throughout in the perfusion experiments.

HFMBs were operated without perfusion as controls for comparison with the perfused system, with medium changed manually on a daily basis. The HFMB culture systems, with or without perfusion, were placed in a 37 °C incubator. The experiments were replicated three times.

The pH, partial pressures of CO_2 and O_2 and concentration of HCO_3^- in the culture medium were measured on days 0, 3, 5, and 7 by using a blood-gas analyzer (IL Synthesis System, Instrumentation Laboratory SpA, Milano, Italy). Lactate production by the cells in the bioreactors was measured by a lactate reagent (Sigma) on days 1, 4, and 7.

Cell proliferation

Cell proliferation was measured by using Alamar Blue™ (Biosource Europe, Belgium) and a fluorescence microplate reader (SPECTRAmax® GEMINI XS Microplate Spectrofluorometer, Molecular Devices Corporation, California, USA) On days 1, 4 and 7, any medium circulation was stopped, medium (2.5 ml) in each bioreactor was removed and replaced by fresh sterile medium containing Alamar

Blue™ (5%). The bioreactor was placed in a 37 °C incubator for 2 hrs before removal and analysis of the medium. The circulation of medium in the perfused group was resumed and fresh medium was added into the non-perfused group.

Cell viability

Cell viability was determined by using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Leiden, Netherlands). After harvesting the bioconstructs (referring to the whole structure accommodated inside the shell of HFMB including hollow fibre membranes, cells and tissue formed within the 3D scaffold of collagen gel and Cytodex 1 microcarriers, see Fig. 6a) from the bioreactor on day 7, one third of the bioconstruct was rinsed in PBS and placed in a 24-well plate. 0.5ml working solution (2 μM calcein and 4 μM ethidium homodimer (EthD-1) in PBS) were added to each well and incubated at 37 °C for 45 mins. Following incubation, several drops of PBS were added to a clean microscope slide. Samples were transferred from the 24-well plate to microscopic slides and viewed under the fluorescence microscope (excitation/emission: calcein 494/517 nm; EthD-1 528/617 nm). Images were oberserved by using a fluorescence microscope (Zeiss Axiphot, Germany) and captured by using software Optimas Version 5.2 (Optimas Corporation).

Histological assessment

After harvesting samples of the bioconstructs from the bioreactors, one third of the samples were rinsed in PBS and fixed in 10% formaldehyde for 15 mins. The samples were embedded in 2% agarose for additional support before routine histological embedding in paraffin. Sections (4 μm thick) were dewaxed by using xylene and stained with haematoxylin and eosin.

Scanning electron microscopy (SEM)

SEM was used to examine the microstructure of the harvested bioconstructs. One third of the bioconstruct removed out of each of the HFMBs (perfused and non-perfused) after *in vitro* culture for 1 week were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer. The samples were dehydrated in ethanol and subjected to critical point drying before mounting on stubs and sputter coating with gold prior to examination in a scanning electron microscope (Philips 505, Eindhoven, NL).

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed t-test and the data are expressed as means ± standard errors.

Results

The pH, HCO_3^- concentration and partial pressure of CO_2 and O_2 of HFMBs on days 0, 3, 5, and 7 are shown in Fig. 2. There were significant differences ($p < 0.05$) between the non-perfused and perfused groups on day 7 for all four of these measurements.

The results of the Alamar Blue™Assay for cell proliferation are shown in Fig. 3A with no significant differences being observed between the non-perfused and the perfused bioreactors on day 1. The cell numbers in the perfused group increased up to 4 days and then stabilized, but the non-perfused group showed a decrease in cell numbers with time. The cell numbers in the perfused group were about 5 times that of the non-perfused group on both days 4 and day 7 and these differences are statistically significant ($p < 0.05$).

The daily lactate production in the culture media of the two groups are shown in Fig. 3B. The amount of lactate produced by live cells is related to cell number and cell activity. Figure 3B shows that daily lactate production in the perfused group increased with time while it decreased in the non-perfused

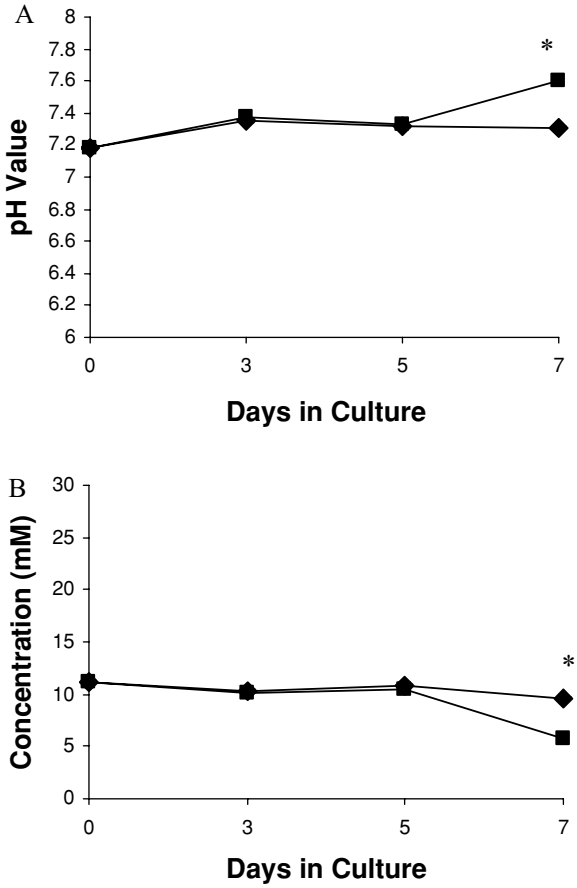

Fig. 2 Analysis of culture medium of HFMB using a blood-gas analyser. A: pH; B: HCO_3^- concentration, C: partial pressure of CO_2, D: partial pressure of O_2. (■: non-perfused; ♦: perfused; $*p < 0.05$)

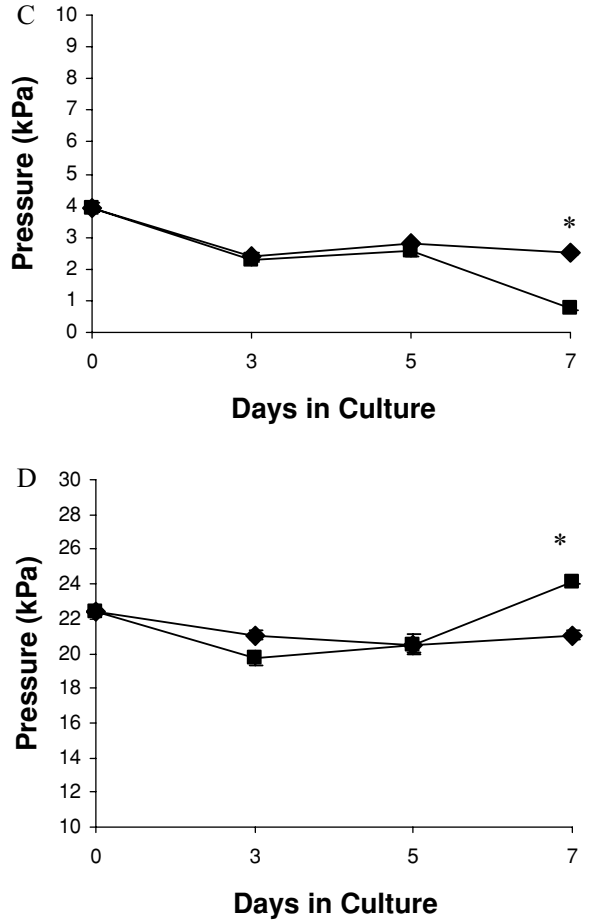

Fig. 2 (*Continued on next page*)

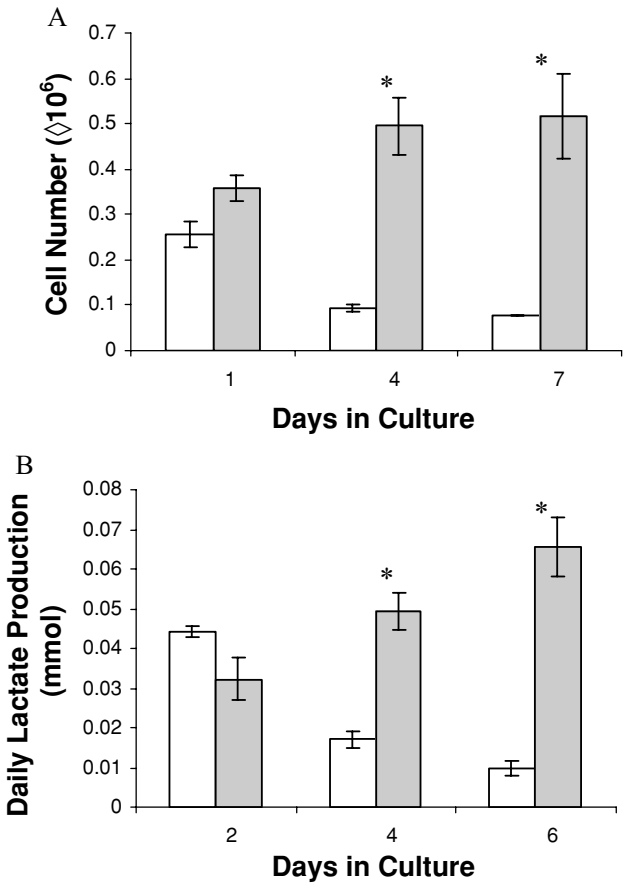

Fig. 3 Cell proliferation A: cell number estimated by Alamar Blue™ assay of non-perfused and perfused groups; B: daily media lactate production in non-perfused and perfused groups. (*$p < 0.05$)□: non-perfused ■: perfused)

group. On both days 4 and 7, the differences between the non-perfused and perfused groups were significant ($p < 0.05$).

The light and fluorescence microscopy of LIVE/DEAD® cell staining of the specimen removed from the HFMBs were shown in Fig. 4. With bright field microscopy, although hollow fibres and microcarriers can be identified, it is difficult to identify individual cells (Fig. 4a and 4b). However, using fluorescence microscopy after LIVE/DEAD® cell staining it is possible to observe the morphology and viability of the RBMFs (Fig. 4c and 4d). In the non-perfused group, the overall cell number was lower compared with the perfusion group (Fig. 4c and 4d). There were many dead cells that stained with red fluorescence in the non-perfused group. In contrast, in the perfusion group, the cells were totally viable as shown by their green fluorescence with no detectable non-viable cells in this group.

In the perfused bioreactor groups, RBMF cells not only covered the surfaces of the microcarriers but also migrated into the collagen gel to form 3D tissue-like structures (Fig. 5b). It was not possible in histologically-stained sections to identify tissue-like structures within the non-perfused groups. In the latter groups, the majority of the RBMF cells appeared to detach from the microcarriers and collagen gels. The few cells remaining on the microcarriers appeared damaged with condensed nuclei and the collagen gel structures showed evidence of disintegration (Fig. 5a).

The overall structure of the bioreactor consisting of the 3D matrix formed by the hollow fibres, microcarriers and collagen gel is shown in Fig 6a. At higher magnifications, it is observed that in the non-perfused group the RBMF cells detach from the microcarrier surface and collagen gels and the collagen gel shows disintegration (Fig. 6b). In contrast, in the perfused group the majority of the surfaces were covered by well-developed flattened fibroblast–like cells with multiple layers of cells (Fig. 6c). In some areas well-organised collagen fibres formed by the RBMF cells were observed.

Discussion

Failure to produce an appreciable mass of any tissues, such as bone, via tissue engineering procedures is due in part to the

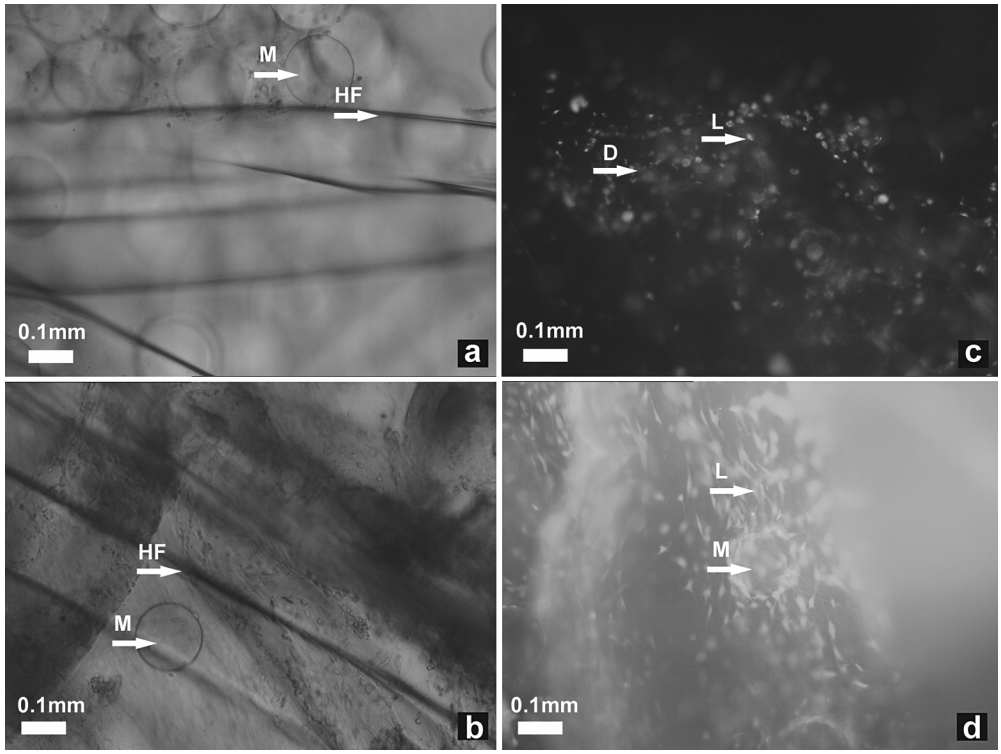

Fig. 4 Light and fluorescence microscopy of HFMBs after culture for one week. (a, b) light microscopy; (c, d) fluorescent microscopy after LIVE/DEAD® staining. (a, c) non-perfused group; (b, d) perfused group. D: dead cell; HF: hollow fibre membrane; L: viablecell; M: microcarrier

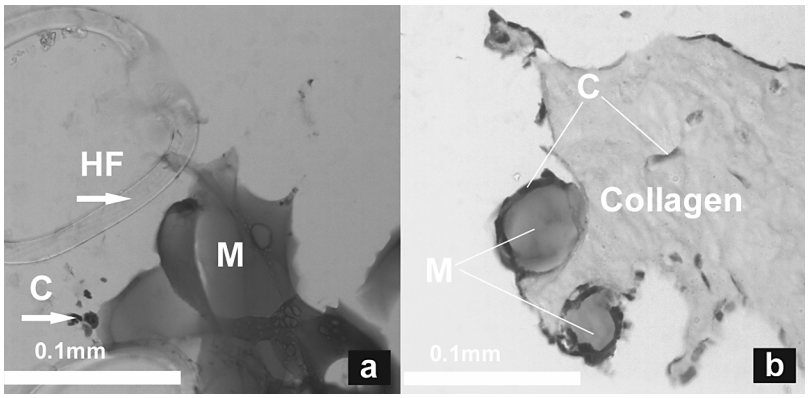

Fig. 5 H&E staining of HFMB construct after culture for one week. (a) non-perfused group: showing RBMF cells detaching from the microcarrier surfaces with only few cells surviving in the collagenous matrix (b) perfused group: showing microcarrier covered by RBMF cells with the cells migrating into the collagenous matrix C: RBMF cells; HF: hollow fibre membrane; M: microcarrier

inability to form a vascular system resulting in limited tissue oxygen and nutrient supply and waste product removal. Cell densities decrease with distance from the construct surface and no cells within a tissue mass can survive being more than approximately 100 μm away from a source of oxygen or nutrients *in vivo* [23]. It has been suggested that the possible maximum thicknesses of engineered cartilaginous, bone-like and cardiac-like tissues grown *in vitro* are approximately 5.0 mm, 0.5 mm and 0.18 mm respectively [3].

To overcome the problem, we propose to develop novel hollow fibre membrane bioreactors, employing semi-permeable hollow fibre membrane networks to distribute nutrients and remove metabolic waste within the mass of a 3D bioconstruct. Hollow fibre membrane bioreactors have been extensively used for high-density mammalian cell culture, [13, 17, 18, 24–27] but to the best of our knowledge this is the first report on its possible application for engineering of structurally organised 3D tissues by using adherent cells. This report presents data that suggests such an objective is feasible.

There were no differences between the cell numbers in the perfused and the non-perfused groups on day 1 ($p < 0.05$). With increasing time, the cell numbers in the non-perfused groups decreased, while in the perfused groups the cell numbers increased showing significant difference on day 4 and 7 between the two groups (Fig. 3A). The cell activity, expressed

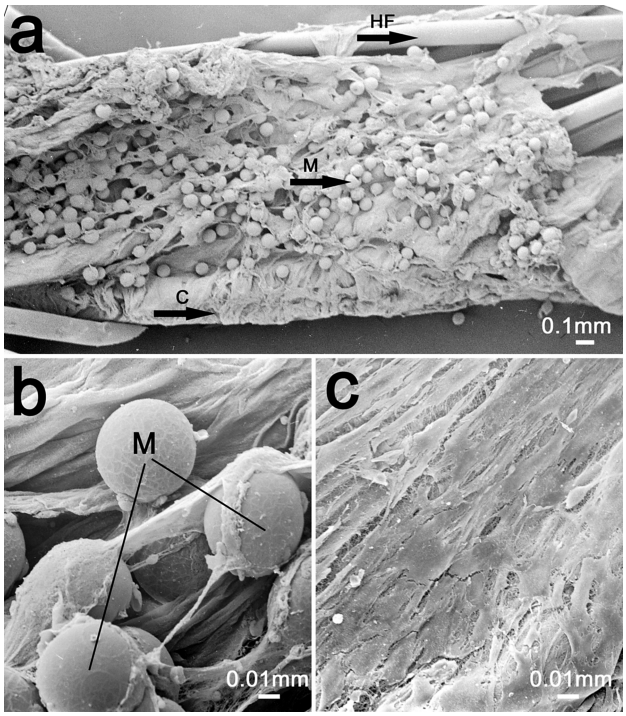

Fig. 6 Scanning Electron Microscopy of HFMBs after culture for one week. (a) General appearance showing hollow fibres, microcarriers and collagenous matrix. (b) Non-perfused group: RBMF cells were detaching from the microcarriers. The collagenous gel matrix appears less intact with exposure of the microcarriers. (c) Perfused group: indicating that the RBMF cells have migrated from the surfaces of the microcarriers and formed multiple layers in the collagenous matrix with formation of newly organized collagen fibres. C: RBMF cells; HF: hollow fibre membrane; M: microcarrier

as daily lactate production, was also significantly higher in the perfused HFMBs compared to the non-perfused HFMBs at these time points. These results indicate the significant advantages of the perfused system for survival and activity of the adherent cells within the scaffold-bioreactor.

Collagen gels have been used as scaffolds for fibroblastic cells in 3D culture systems [28], but fibroblasts naturally contract in such systems and this causes shrinkage of the collagen gel and a sharp decrease in volume [29]. In an attempt to overcome this problem in the present study, cells were initially grown on Cytodex 1 microcarriers These are cross-linked dextran polymer beads of 190 μm average diameter which are positively-charged allowing the culture of anchorage-dependent cells. Using microcarriers can greatly enhance the surface to volume ratio of cell culture and microcarriers are often used in high density cell culture. Cytodex 1 microcarriers were used initially for cells to attach and proliferate. After mixing with the collagen gel, the fibroblastic cells migrated into the collagen gel and reduced contraction was observed. Secondly, the presence of porous microcarriers can enhance the porosity of the collagen gel, thus increasing the diffusion efficiency of oxygen and nutrients in the 3D scaffold [30]. In addition, Cytodex 1 microcarriers can help maintain the structural integrity of the 3D construct during tissue development. The combination of the microcarriers and the collagen gel would appear to have been successful in the present study since there is evidence of the migration of the RBMF cells from the microcarriers and their integration into the collagen gel to form 3D neo-tissues.

In all the assays performed (lactate production, cell viability and cell proliferation), it was observed that, whilst there were no significant differences on day 1, there was significantly improved cell survival and proliferation in the perfused bioreactors at later time points (days 4 and 7). This was confirmed by the light and electron microscopic examinations showing increased numbers of intact cells invading the ECM in the perfused samples compared to the cell debris observed within the non-perfused samples. These observations are consistent with the proposals that continuous perfusion provides a more efficient supply of nutrients and also a stable pericellular microenvironment compared to the non-perfused group, where diffusion from the outer surface will be a limiting factor [20].

Further development of hollow fibre membranes with controlled biodegradable characteristics will be valuable for future work. The rates of fibre biodegradation could be adjusted according to the rate of tissue formation or to the vascular invasion after implantation *in vivo*. With time, this would result in increased pore sizes of the hollow fibre membranes, thus providing more nutrient and oxygen supplies to meet the needs of increasing cell densities and tissue formation. It is very important to match the degradation rate of the hollow fibre membrane with the growth of the cells and tissues. Cellulose acetate hollow fibre membranes were used in the present studies as proof of concept. However, more advanced biomaterials with controllable degradation rates are being reported [31, 32], and thus it is feasible that suitable biodegradable hollow fibre membranes may become available in the near future. In addition, the composition of the composite scaffold needs further investigation and development with possible substitution of biodegradable materials for adherent cell growth for the microcarrier particles and alternatives to the collagen scaffolds used here.

In conclusion, perfusion via hollow fibres in the membrane bioreactor combined with microcarriers and collagen has been shown to significantly improve adherent cell survival in 3D structures. The results from a variety of physiological indicators show significantly better survival and proliferation of RBMF cells in the perfused group compared to the non-perfused group after 7 days of *in vitro* culture. Further work is required to evaluate the longer term effects on tissue development and cell survival within such a perfused bioconstruct. As in all tissue-engineered constructs, subsequent consideration of methods to integrate the tissue mass produced with the host systemic circulation is a key issue.

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