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Human liver microtissue spheroids in hollow fiber membrane bioreactor

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ABSTRACT

The aim of this work was to create human liver microtissue spheroids metabolically active by using a hollow fiber membrane bioreactor whose design and structural features ensure a uniform microenvironment and adequate oxygenation. Human hepatocyte spheroids with uniform size and shape were formed through self-assembling and cultured into the bioreactor. Adjacent spheroids fused, giving rise to larger microstructures around the fibers forming liver-like tissue, which retained functional features in terms of urea synthesis, albumin production, and diazepam biotransformation up to 25 days. The overall data strongly corroborates that within the bioreactor a proper oxygenation and supply of nutrients were provided to the cells ensuring a physiological amount even in the spheroids core. The oxygen uptake rate and the mathematical modelling of the mass transfer directly elucidated that liver microtissue spheroids are not exposed to any oxygen mass transfer limitation. The minimum oxygen concentration reached at the center of multiple spheroids with diameter of 200 μm is significantly higher than the one of the perivenous zone *in vivo*, while for larger microtissues (400 μm diameter) the oxygen concentration drops to values that are equal to the maximum concentration found in the liver periportal zone. Both experimental and modelling investigations led to the achievement of significant results in terms of liver cell performance. Indeed, the creation of a permissive microenvironment inside the bioreactor supported the formation and long-term maintenance of functional human liver microtissues.

1. Introduction

Acute liver failure and drug development assays require the availability of cells capable of expressing all human liver-specific functions. The expression of several drug metabolizing isozymes cytochrome P450 (CYP450), as well as nitrogen excretion and albumin synthesis, is usually downregulated in hepatoma or immortalized cell lines [1,2] and hepatocytes from animals have an inherent limitation due to the differences in the metabolic pathways between species. For the study of liver physiologic and pathological phenomena, it is of primary importance to have a faithful *in vitro* model that resembles as much as possible the *in vivo* conditions.

Primary human hepatocytes are considered the gold-standard for bio-artificial liver devices, drug development, and toxicity tests. Yet, although primary hepatocytes are known to maintain most of their liver-specific functions immediately after isolation, they lose these functions in 2D culture systems [3–5]. An improvement in the *in vitro* long-term maintenance of liver functions has been achieved by developing 3D culture models such as collagen sandwich and spheroids [6–8]. These systems provide a microenvironment in which cell-cell and cell-matrix interactions are maximized mimicking the *in vivo* physiological liver architecture. However, the collagen sandwich model includes some drawbacks due to the batch-to-batch variation and the scaling-up that make it difficult to use. An interesting approach that circumvents these problems is to culture hepatocytes in 3D spheroids obtained by a self-assembling process. The use of hepatocyte spheroids has been shown to increase liver-specific functions in cells owing to the high degree of intercellular contacts that are crucial for communication of signals and integration of gene and metabolic patterns [9,10]. In particular, hepatocytes grown as spheroids were shown to maintain albumin production, urea synthesis, and biotransforma-

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tion activity for longer time periods than monolayer and collagen sandwich cultures [11,3]. This is likely due to the 3D cytoarchitecture, and a more realistic interaction between cells and the matrix. Additionally hepatocyte spheroids have the potential to be employed for the fabrication of *in vitro* liver tissue through a fusion process. Several methods that have been used for generating 3D aggregates include the hanging drop method [12], rotational bioreactors [13], microfabricated microwells [14], selectively-adhesive patterned structures [15], and the liquid overlay method [16]. Hepatic long-term functional and toxicological investigations by means of tissue like spheroids have been widely attempted. Recently, a 3-D primary human hepatocytes spheroids system was developed [17] highlighting important achievements in terms of phenotypic and morphologic retention as well as for specific hepatic functions for spheroids of a consistent size (about 200 μm). As corroborated by Curcio et al. [18], spheroids with size exceeding 200 μm, in gas impermeable systems suffer severe oxygen limitation in most of their structure, attaining the lowest partial pressure of 12 mmHg in the core. Therefore, in many bioartificial liver devices the absence of a vascular network in large spheroids may eventually cause mass transfer limitations of oxygen that lead to an impairment of cell viability and functions. Indeed, a reduced cell survival (60%) due to the insufficient oxygen supply remains an issue for spheroids even when hepatocytes establish direct cell-cell interactions in co-culture with human umbilical vein endothelial cells (HUVECs), which should ensure an optimal tissue-like reorganization [19]. Dynamic systems such as hollow fiber membrane bioreactors can effectively contribute to accomplish the challenging goal of sustaining and maintaining hepatocyte spheroids with sufficient amount of nutrients, including oxygen. An ideal hepatocyte spheroid model should also allow fluid flow to improve mass transfer. Due to a continuous nutrient supply and waste removal, perfusion cultures show an improved viability, life span, and metabolic performance on primary hepatocytes. However, spheroids cultured in systems under a direct perfusion are exposed to the momentum generated by Stokes force, which determines their detachment and affects their aggregation [20]. Indeed, perfusion-stirred tank bioreactors for culturing human primary hepatocytes as spheroids minimize the gradient of soluble factors in the culture bulk [8], but do not prevent shear stress generated by fluid flow, which negatively affects the hepatocyte functions.

To overcome many limitations that currently exist in bioreactor systems that could hinder their use as *in vitro* systems for studying liver functions, liver disease, and testing new drug cytotoxicity, here we investigated the long-term maintenance of human hepatocyte microtissue spheroids in a crossed hollow fiber membrane bioreactor. The main distinguishing feature of this bioreactor, which is based on a concept developed previously [21], is the use of two bundles of hollow fiber (HF) membranes in polyethersulfone (PES): one bundle of feeding fibers to provide oxygen, nutrients and metabolites to the cells and one bundle of removing fibers to eliminate catabolites and specific products from cell compartments. The fibers are cross-assembled in an alternating manner, separated from each other by a distance of 250 μm in order to establish two intraluminal compartments in which the medium flows, and an extraluminal compartment in which microtissue spheroids are cultured and protected from shear stress. The bioreactor chamber creates a homogeneous microenvironment in which the continuous exchange of fresh and depleted media ensures the proper balance between the amount of oxygen supply and the consumption by cells, thus preventing hypoxia and necrosis within the spheroid.

The challenge pursued with this work is the creation of microtissue spheroids into a bioreactor that ensures a continuous perfusion preserving uniform levels of nutrients, hormones, and endogenously secreted regulators throughout culture time. By producing a shear stress-free microenvironment with continuous feeding, no mass transfer limitations, three-dimensional cell interactions, and fully controlled culture properties, this system creates a permissive environment for the formation of human liver organoids and the long-term maintenance of liver-specific activities.

The scientific results achieved make the proposed system a robust tool for screening tests that require long-term culturing of hepatocytes, which passed the currently available high-throughput short-term screening systems.

2. Materials and methods

2.1. Bioreactor

The bioreactor consists of 180 PES HF membranes used for the medium inflow and outflow. The two fiber systems were assembled in an alternating manner and potted with polyurethane adhesive (BASF Polyurethane GmbH, Lemfoerde, Germany) within glass housing (Fig. 1). The fibers were potted at each end in order to establish three separate compartments: two intraluminal compartments within the inlet and outlet PES fibers, and an extraluminal compartment or shell outside of the fibers. The intraluminal and extraluminal compartments communicate through the pores in the fiber wall.

The bioreactor (volume: 30 ml) is connected to a perfusion circuit consisting of micro-peristaltic pump, gas-permeable silicone tubing reservoir of medium and oxygen sensors interfaced via an optic fiber to the data recording unit.

The oxygenated medium enters from the reservoir to the membrane bioreactor with a flow rate Q_f of 0.8 ml/min that was set on the basis of average retention time. Fresh medium was perfused in single-pass and the stream leaving the bioreactor Q_{out} was collected as waste until approaching the steady state. When the system reached the steady state, the stream leaving the bioreactor was recycled (Q_r) in order to obtain the accumulation of products.

2.2. Cell culture

Cryopreserved primary human hepatocytes (Life Technologies, California, US), isolated from human tissue were thawed in a 37° C water bath with gentle shaking. Cell suspension was slowly transferred into pre-heated 25 ml of Williams' medium E (without phenol red) supplemented with dexamethasone and a cocktail solution of fetal bovine serum, penicillin-streptomycin, human recombinant insulin, GlutaMAX™ and HEPES, and then centrifuged at 50*g* at room temperature for 5 min. The viability of the hepatocytes assessed by Trypan blue exclusion was around 85%.

For the formation of hepatocyte spheroids an agarose mold was fabricated by using a 3D Petri Dish® micro-mold (Sigma) consisting of 256 circular recesses (16 × 16 array) with diameter of 400 μm and depth of 800 μm. Molten agarose at 2% (500 μl) was cast on each of the micro-molds and left for 10 min to set. Then the agarose molds were removed and placed in 12-well plates.

The human hepatocytes were then seeded in 256-well agarose molds at a density of 3×10^5 cell/mold. To promote cell aggregation into spheroids, medium was supplemented with 10% FBS; cells were cultured in this medium for 5 days.

Spheroids (6800 ± 100) were then seeded into the HFMBR on the outer surface of PES HF membranes previously sterilized and conditioned with medium containing 10% FBS, dexamethasone, a cocktail solution of penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, BSA, and linoleic acid), GlutaMAX™, and HEPES (Fig. 1). Afterwards, the FBS content in the medium was decreased gradually reaching a serum-free medium at day 17. The experiments lasted for 24 days. The bioreactors were incubated at