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In vitro evaluation of a multi-layer radial-flow bioreactor based on galactosylated chitosan nanofiber scaffolds

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ABSTRACT

Clinical use of bioartificial livers (BAL) strongly relies on the development of bioreactors. In this study, we developed a multi-layer radial-flow bioreactor based on galactosylated chitosan nanofiber scaffolds and evaluated its efficacy in vitro. The bioreactor contains 65 layers of stacked flat plates, on which the nanofiber scaffolds were electrospinned for hepatocyte immobilization and aggregation. Culture medium containing pig red blood cells (RBCs) was perfused from the center to periphery, so that exchange materials are sufficient to afford enough oxygen. We determined the parameters for hepatocyte-specific function and general metabolism and also measured the oxygen consumption rate (OCR). Microscope and scanned electron microscopy observation showed a tight adhesion between cells and scaffolds. Compared with the control (bioreactors without nanofiber scaffolds), the number of adhered cells in our bioreactor was 1.59-fold; the protein-synthesis capacity of hepatocytes was 1.73-fold and urea was 2.86-fold. Moreover, the OCR of bioreactors with RBCs was about 1.91-fold that of bioreactors without RBCs. The galactosylated chitosan nanofiber scaffolds introduced into our new bioreactor greatly enhanced cell adhesion and function, and the RBCs added into the culture medium were able to afford enough oxygen for hepatocytes. Importantly, our new bioreactor showed an exciting efficiency, and it may afford the short-term support of patients with hepatic failure.

1. Introduction

Orthotopic liver transplantation is a unique effective treatment for end-stage liver diseases [1]. However, due to severe donor-liver shortage, high cost and exacerbation of disease, many patients die before they can receive the operation. Therefore, bioartificial liver (BAL) has been proposed as a temporary liver support for patients awaiting liver transplantation [2].

Nevertheless, the clinical application of this new treatment strategy is not very optimistic [3]. One major reason is that hepatocytes rapidly lose liver-specific functions and viability when cultured in the bioreactor in vitro. Thus, to build a good bioreactor, it is crucial to mimic the microenvironment of hepatocytes in vivo and to afford the hepatocytes in a suitable environment [4].

In vivo, most cells adhere to the extracellular matrices (ECMs), which have extremely complex topography in the nanometer range. In order to mimic the topography of ECMs, various materials have been fabricated into nanometer materials [5] and they can affect cell migration, adhesion, proliferation and other cellular behaviors [6,7]. Nanometer scaffolds have been widely used in different tissue engineering, such as bone [8], nerve [9] and bladder [10]. However, there have been few reports on the application of nanometer scaffolds in the liver tissue engineering. We reported the influence of chitosan nanofibers on hepatocytes in vitro in a previous study, in which we showed that nanofiber scaffolds mimic ECMs well and enhance cell adhesion [11].

Besides the nanometer topography of ECMs, there are many important biochemical groups on the ECMs, which are also essential for the growth of cells, such as the galactose ligands. Many studies have shown that the asialoglycoprotein receptors (ASGPR) on the surface of hepatocytes selectively adhere to galactose ligands [12–14]. This interaction between ASGPR and galactose ligands can induce the formation of hepatocyte aggregates [15,16], which exhibits a higher level of liver-specific functions [17]. Therefore, intensive efforts have been made in developing new scaffolds modified with galactose ligands to enhance hepatocyte adhesion as well as to maintenance of liver-specific functions and

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mechanical stability [18–20]. We grafted the galactose ligands onto the chitosan nanofibers in the previous study and observed its influence on hepatocytes cultured in vitro. Our results showed that compared with hepatocytes on chitosan nanofiber scaffolds and galactosylated chitosan film, hepatocytes cultured on galactosylated chitosan nanofiber scaffolds exhibit excellent cell bioactivity and higher levels of liver functions for a long time [21].

In this study, we developed a multi-layer radial-flow bioreactor. We introduced nanofiber scaffolds into the bioreactor to mimic the topography of ECMs, and grafted the galactose onto nanofibers to mimic the biochemical environment of ECMs. Moreover, we added red blood cells (RBCs) into the medium to mimic the arterial blood in order to meet the high oxygen demand of hepatocytes. We hypothesize that nanofiber scaffolds could enhance the cell adhesion by tight contact between cells and scaffolds; the grafted galactose group could enhance the hepatocellular functions by the formation of spheroids; and the RBCs added into the medium could improve the oxygen supply to hepatocytes.

2. Materials and methods

2.1. Animals and reagents

Outbred white pigs with a weight of 15–20 kg received humane care. All animal procedures were performed according to institutional and national guidelines and approved by the Animal Care Ethics Committee of Nanjing University and Nanjing Drum Tower Hospital. RPMI 1640 were purchased from GIBCO (USA). Lactobionic acid (LA) and chitosan (low molecular weight, brookfield viscosity 20,000 cps, 85% deacetylation) were purchased from Sigma–Aldrich (Saint Louis, USA). *N*-Hydroxysuccinimide (NHS) was purchased from Thermo-Pierce (Rockford, USA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and *N*,*N*,*N*′*N*′-tetramethylethylenediamine (TEMED) were obtained from TCI (Tokyo, Japan). Poly(ethylenoxide) (PEO, $M_W \approx 1 \times 10^6$) was supplied by Guoren Chemical Co. (Beijing, China). All other reagents were of analytical reagent grade.

2.2. Cell preparation

Primary hepatocytes were harvested by a two-step in situ collagenase perfusion technique as previously described [22]. The viability of isolated hepatocytes was determined by trypan blue exclusion, which was more than 95%.

2.3. Bioreactor configuration

The multi-layer bioreactor consisted of a housing, a hollow column stent, and stacked flat plates, all of which were made of polycarbonate (PC). The fully-assembled bioreactor contained a stack of 65-layer round flat plates, on which galactosylated chitosan nanofiber scaffolds were electrospun for hepatocyte immobilization and aggregation. The diameter and the thickness of each plate were 10.4 cm and 1 mm, respectively. There was a hole with the diameter of 1 cm in the center of each plate, which was used to fix the plates onto the stent. The channel height between every two neighboring plates was maintained at 0.5 mm with the spacers attached to the bottom of each plate. The stent was open on the top and closed at the bottom, with four vertical-side holes on the lateral wall, which were broken up into eyelets between every two plates. The last component was a housing, which was a cylindrical container with an outlet on the bottom. When the stacked plates were fixed onto the stent, they were put into the housing, and the lid with an O-ring was screwed onto the housing in order to provide a water tight seal. The culture medium entered the bioreactor from the top opening of stent, then flowed onto the surface of each flat plate through eyelets, and finally flowed out from the outlet of housing. The height of this bioreactor was about 10 cm, and the effective volume was 480 ml.

2.4. Electrospinning of galactosylated chitosan on flat plates

Coupling of chitosan with LA was performed by an active ester intermediate with EDC and NHS as previously reported [23]. Then the galactosylated chitosan solution was prepared by dissolving galactosylated chitosan powder into the solvent of formic acid/ethanol (volume ratio = 7:3) at a concentration of 5 wt%. Subsequently, 0.5 wt% of PEO was added into the above solution. The stock solution was then filled into a 5 ml glass syringe fitted with a 20G needle and then expressed at 5 ml/h with a syringe pump. The nanofibers were collected on the flat plates at a fixed distance of 10–20 cm from the needle tip.

2.5. Experimental set-up

Six bioreactors were divided into two groups: an RFB-nano group ($n = 3$), in which hepatocytes were cultured in bioreactors with nanofiber scaffolds on the surface; and an RFB-control group ($n = 3$), in which hepatocytes were cultured in bioreactors without nanofiber scaffolds. Freshly-isolated pig RBCs (1.2×10^{11}) were repeatedly washed before being added into the circulated medium of these bioreactors, and the final concentration of RBCs was 2.5×10^{11}/L, which is equal to 5% of that of the human body. In order to investigate the oxygen supply function of RBCs, an Oxy-control group was also included ($n = 3$), in which no RBCs were added into the bioreactors. The oxygen consumption rate (OCR) of RFB-nano and Oxy-control were examined and compared. The function of bioreactors was tested on d 1, d 2 and d 3 after cell loading.

2.6. Bioreactor procedure

In our study, 1×10^{10} freshly isolated porcine hepatocytes were pre-mixed in 480 ml RPMI 1640 culture medium containing 10% BSA, 0.5 mg/ml insulin, 10 mM $NaHCO_3$, 50 mg/ml penicillin and streptomycin, and 100 mg/ml neomycin. Then, the medium was filled into the bioreactor by a peristaltic pump (JHBP-2000B, Guangzhou, China). The whole bioreactor was incubated for 1 h at 37 °C and 5% CO_2 until the cells were adhered onto the surface of plates.

After the cells were adhered to the plate surface, the bioreactor was connected with the culture medium circuit containing 1.2×10^{11} RBCs. Culture medium in the bioreactor was pumped into an oxygenator (Affinity, USA) through Fresenius silicone tubing (Fresenius, Germany) by a peristaltic pump and was circulated at 100 ml/min. The gas circuit of oxygenator was connected to an oxygen tank. After sufficient gas exchange in the oxygenator, the medium entered bioreactor again through the top opening of stent, and then flowed over the cells cultured on the surface of plates, which not only provided the hepatocytes with oxygen and nutrient, but also removed metabolic products.

2.7. Bioreactor tests

Bioreactor tests included metabolic function, damage evaluation, morphological observation, oxygen supply and cell quantification. Culture medium was collected every day for function determination. The metabolic analysis consisted of albumin synthesis, urea production, ammonia elimination and carbohydrate metabolism. The albumin and urea were tested by ELISA kits (Bethyl Laboratories Inc, USA) according to the operation manual, and the others were tested with an automated chemical analyzer (MEGA Toshiba, Japan). Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined as damage factors, which were also tested by an automated chemical analyzer (MEGA Toshiba, Japan). The oxygen consumption was determined by the blood gas analyzer as previously described [24]. The morphology was observed by both microscope and scanned electron microscopy (SEM) after the experiment. We collected the culture medium at the end of experiments and counted the hepatocytes directly by a hemocytometer. Thus, we could obtain the number of free cells and calculated the quantity of adhered cells.

2.8. Statistic analysis

Data were presented as mean ± SD. Statistical analyses were performed with the *t*-test. A two-tailed *P* value <0.05 was considered statistically significant. All data were analyzed with the SPSS software (version 11.0).

3. Results

3.1. Liver-specific functions

We determined albumin and urea production as well as ammonia elimination and carbohydrate metabolism to assess the liver-specific functions (Fig. 1). Albumin production initially increased from 1 d to 2 d in both groups; and then it showed a decreasing tendency in RFB-Ctr but remained almost constant in RFB-nano (1.73-fold higher than that of RFB-Ctr on 3 d, $P < 0.05$). Urea production was equal in the first day in both groups; and then it remained stable in RFB-Ctr but increased in RFB-nano over 3 days (2.86-fold higher than RFB-Ctr at d 3, $P < 0.05$). The ammonia elimination of RFB-Ctr was slightly higher than RFB-nano on d 1, but it gradually increased 1.42-fold for RFB-nano and decreased to 56.7% for RFB-Ctr on d 3. There was a significant difference between the two groups on d 3 ($P < 0.05$). We also determined the glucose consumption as the parameter for carbohydrate metabolism. After an initial phase of glucose production, both groups began to