Radial Flow Type Bioreactor for Bioartificial Liver Assist System Using PTFE Non-Woven Fabric Coated with Poly-amino Acid Urethane Copolymer

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Summary: Recently, various types of hybrid bioartificial liver using the porcine hepatocytes have been widely studied but their clinical usage is limited by various difficulties. One of the most efficient bioreactor is the radial flow system equipped with silicon oxygenator. Here, our bioreactor system provides the oxygenator and the radial flow of medium that supplies the nutrient efficiently. The adhesion and growth of the cultured hepatocytes are greatly improved by using polytetrafluoroethylene (PTFE) non-woven fabrics coated with poly-amino acid urethane copolymer (PAU). Its functional activities were established after being kept for one week at the high value in this bioreactor system. These results indicate that, PTFE non-woven fabric and radial flow technique is proven to be the best bioreactor for a hybrid artificial liver support system (HALS).

Keywords: adhesion; HALS; poly-amino acid urethane copolymer (PAU); porcine hepatocytes; tissue engineering

Introduction

The hybrid artificial liver support system (HALS) is expected to become an effective treatment as bridge for patients with severe liver failure, who are waiting for liver transplantation.[1] Various types of HALS using cultured hepatocytes have been tested by animal model, and the results of these experiments show that it is not easy to maintain the liver specific function of hepatocytes during operating the HALS.[2–6] Only a few groups have performed clinical trials using a hollow fiber bioreactor type HALS, which has the advantage of immunoisolation from xenogenic hepatocytes.[7–9] However this model has some difficulties in building scale-up, the hollow fiber bioreactor or a conventional packed-bed bioreactor may suffer from stacking of hepatocytes upon each other and the weak flow of culture medium that may cause cell death.

A radial flow bioreactor has been proposed and further investigated in order to resolve these problems. This bioreactor consists of a vertically extended cylindrical PTFE non-woven fabric coated with PAU scaffold through which the culture medium flows continuously from periphery towards the central axis. The radial flow will generate a beneficial concentration gradient of oxygen and nutrients, while preventing excessive stresses and buildup of waste products. In addition, PAU is the block copolymer that consists of a small amount of poly (γ-methyl-L-glutamate) (PMLG)

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and the polyurethane. The urethane segments are hydrophobic and also actively interact with the other hydrophobic materials such as PTFE, and the PMLG segments with the α-helix structure that possess the cytocompatibility. Furthermore, PAU has been firmly coated onto the PTFE fiber and acts as an artificial extracellular matrix. Thus, the radial flow bioreactor system with PTFE non-woven fabric coated with PAU is good for high density and large-scale cell cultured with long-term viability.

In this study, the hepatocytes were isolated from slaughtered porcine, so as to be cultured in the radial flow bioreactor system for HALS. The effectiveness of this system regarding on the activity and maintenance of the hepatocytes functions were examined.

Materials and Methods

Isolation of Hepatocytes

By using our method, hepatocytes were isolated from a lobe (about 84 g) of liver of slaughtered adult pig by perfusion technique utilizing dispase and collagenase. The total amount of over 2.6×10^9 hepatocytes were routinely obtained from a lobe. Hepatocytes of more than 90% viability, determined by trypan blue exclusion method were used for the experiments.

Perfusion Culture in Radial Flow Bioreactor System

Perfusion culture experiments were performed in a radial flow bioreactor system at 37 °C. The culture medium was composed of WE medium supplemented with 5%(v/v) fetal bovine serum (Sigma, USA), 0.01 μmol/l insulin(Wako Pure Chemical Industries, Ltd., Japan), 0.2 μmol/l dexamethasone (Wako Pure Chemical Industries, Ltd., Japan), 5 μg/l epidermal growth factor (Wako Pure Chemical Industries, Ltd., Japan), 10^5 U/l penicillin(Sigma, USA), 0.1 g/l streptomycin (Sigma, USA) and 1.5 mmol/l L-ascorbic acid phosphate (Wako Pure Chemical Industries, Ltd., Japan).

Figure 1 shows the schematic diagram of a radial flow bioreactor system. The radial flow bioreactor consists of a PTFE non-woven fabric coated with PAU and hollow fiber. Hepatocytes suspension (2.0×10^9 in 50 ml) was inoculated into a medium-preparative tank, and then the medium was perfused to the bioreactor from the medium-preparative tank at a flow rate of 17 ml/min for 10 minutes. Subsequently, the medium was circulated at 84.2 ml/min during the culture experiments. A mixed gas containing air, oxygen and carbon dioxide was introduced into the medium-preparative tank through a control equipment and computer to maintain pH value at 7.3 and diluted oxygen (DO) at 313 μmol/l. The medium exchange was performed every day throughout the culture period.

Measurement of Hepatocytes Functions

Ammonium Metabolism Rate and Albumin Secretion Rate

To assess the ammonium metabolism of the cultured hepatocytes, 1 mmol/l NH_4Cl was supplemented into the medium after the medium exchange. Ammonium concentration was measured at 0, 3, and 6 hours after ammonium-loading using a commercially available kit (AMICHEK^{TM} meter; Arkray Factory Inc., Japan). The medium sample was taken before and after the exchange of medium for albumin secretion measure. Albumin secretion was measured by enzyme-linked immunoabsorbant assay, (ELISA).

Glucose Consumption Rate and Oxygen Consumption Rate

The glucose level was analyzed with a commercially available assay kit (glucose C2 test Wako Pure Chemical Industries, Ltd., Japan). The oxygen uptake rate was estimated by measuring DO at the inlet (medium-conditional vessel, Figure 1 (7)) and the outlet (between 10 and 11 in Figure 1) of the cell adhesive radial flow bioreactor. Measured DO data was recorded by the computer (5, in Figure 1).

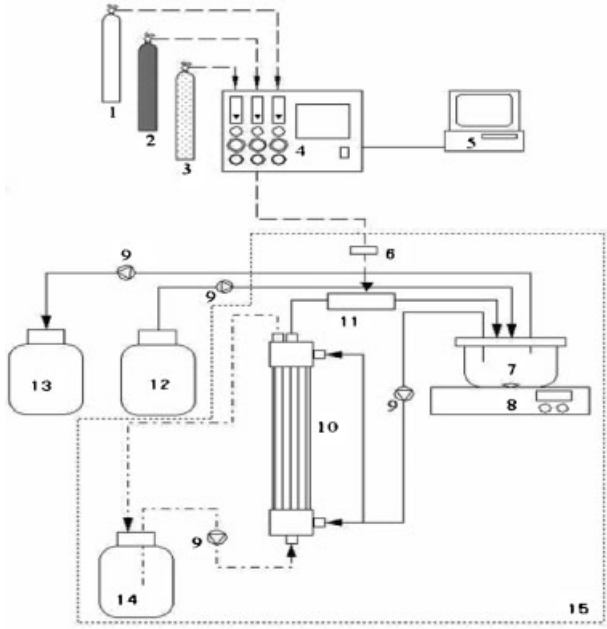

Figure 1.
A schematic diagram of radial flow bioreactor for artificial liver assists system.(1) CO_2 bomb (2) O_2 bomb (3) Air bomb (4) Control equipment (5) Computer (6) Membrane-filter (7) Conditioning vessel (8) Stirrer (9) Peristaltic pump (10) Radial flow bioreactor module (11) Silicone module (12) Fresh medium tank (13) Spent medium tank (14) Plasma bottle (15) Incubator.

Scanning Electron Micrograph (SEM)
Hepatocytes attached on the culture PAU coated PTFE were fixed with 2.5% glutaraldehyde, and dehydrated with graded ethanol (50, 60, 70, 80, 90, 95 and 99.5%). The specimens after critical point drying with carbon dioxide (Drier EMITECH K-850; Meiwa Shoji Co., Ltd., Japan) were coated with palladium by sputtering (plasma multi coater PMC-5000, Meiwa Shoji Co., Ltd., Japan) and then were subjected to SEM observation (SM-300, Topcon Co., Japan).

Histological Study
After electrophysiological study, the transverse section (2 μm thick) from scaffold was taken. The section was stained with hematoxylin & eosin (H&E) staining, as well as azan staining to demonstrate esophageal carcinoma tissue group and was examined by light microscopy (DMR, Leica Co., Japan).

Results

Ammonium Metabolism Rate and Albumin Secretion Rate
The ammonium metabolizing activity and secreting albumin activity of the hepatocytes were established after being kept for one week at the high value in the bioreactor system as shown in Figure 2 and Figure 3.

The hepatocyte functions showed stable ammonium metabolic rate 75.8 μmol/reactor/h to 132.4 μmol/reactor/h and stable albumin secretion rate; 8.6∼24.3 mg/reactor/h after 1 week.

Glucose Consumption Rate and Oxygen Consumption Rate
The glucose consumption rate has been increasing from 0.16 g/reactor/day to 0.73 g/

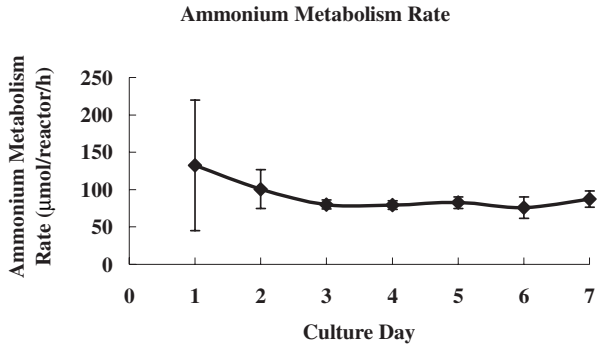

Figure 2.
Mean ammonium metabolic rate of the hepatocytes cultured in radial flow for 7 days.

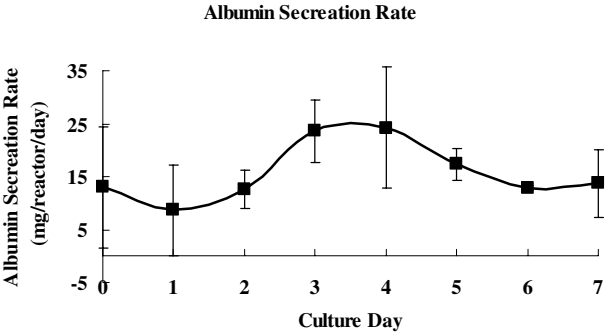

Figure 3.
Mean albumin secretion rate of the hepatocytes cultured in radial flow bioreactor for 7 days.

reactor/day as shown in Figure 4, whilst the oxygen consumption rate has also been increasing from 475 μg/reactor/min to 630 μg/reactor/min as shown in Figure 5. These results indicate that hepatocytes proliferate properly with the increasing of respiration rate and energy produced during 7 days culture.

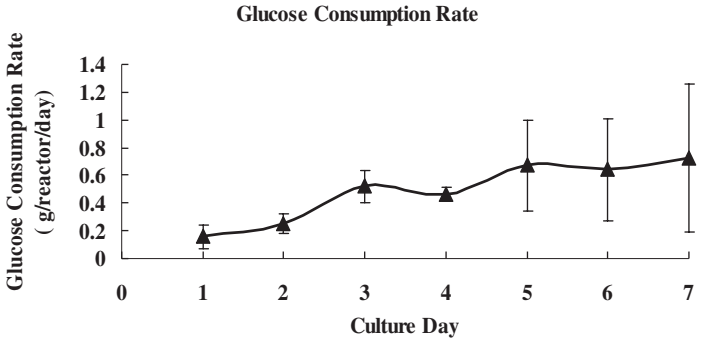

Figure 4.
Mean glucose consumption rate of the hepatocytes cultured in radial flow bioreactor for 7 days.

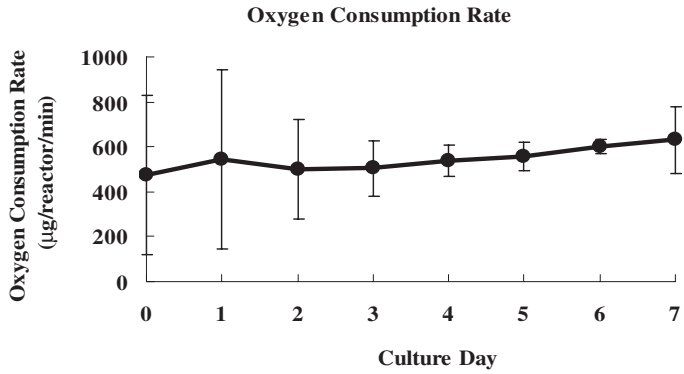

Figure 5.
Mean oxygen consumption rate of the hepatocytes cultured in radial flow bioreactor for 7 days.

SEM and Histological Observation

Figure 6 shows the SEM observation of PTFE non-woven fabric homogeneously coated with PAU before the perfusion culture experiment. The porous structure of PTFE non-woven fabric is maintained and the fabric is changed into hydrophilic and cell adhesive one. Figure 7 shows the SEM observation of the hepatocytes cultured in the radial flow bioreactor after 7 days of culture experiment. Large amount of hepatocytes were aggregated, and adhered onto the surface of PTFE coated with PAU. In addition, the hepatocytes were covered with extracellular matrix (ECM)-like layer. These were proven by H&E and Azan staining as shown in Figure 8. H&E staining pictures show many hepatocyte cells. Azan staining pictures show the existence of connective tissue including collagen.

Discussion

In this study, the activities of ammonia metabolism of hepatocytes were significantly 200 times higher than that of hepatocytes cultured in radial flow bioreactor consists of porous glass bead microcarriers that we have conducted earlier

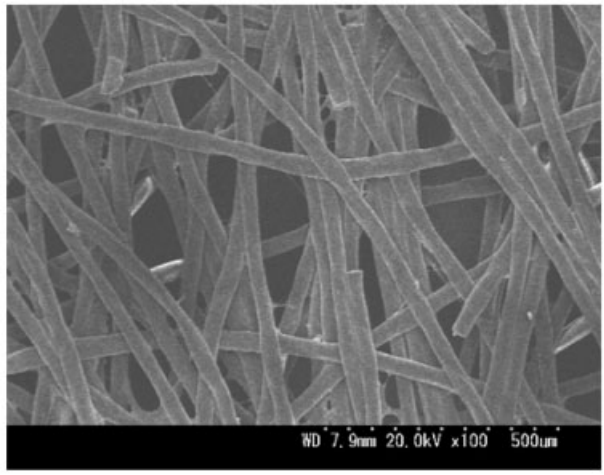

Figure 6.
SEM micrograph of PTFE coated with PAU (×100).

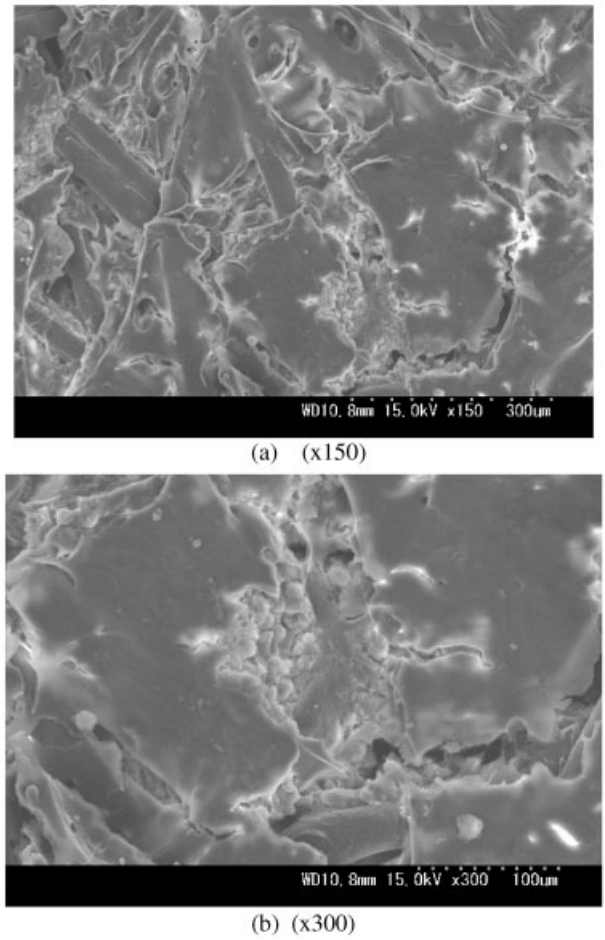

Figure 7.
SEM micrograph of cultured in radial flow bioreactor. (a) (×150), (b) (×300).

(data not shown). This result suggests that by enhancing the immobilized hepatocytes on the PTFE non-woven fabric coated with PAU may also enhances the functional activities of hepatocytes as shown in Figure 2 and Figure 3. Uchida et al.[27,28] had demonstrated that coating with PAU hydrophobic PTFE been changed to hydrophilic PTFE but still maintaining the porous structure. PAU consists of the block copolymer segments of urethane, with a small amount of PMLG at the center of the copolymer chain, where most of the PMLG accumulate at both terminals of the copolymer chain. The urethane segments have good adhesion to other materials, and the PMLG segments have the α-helix structure of protein which posses the high cytocompatibility as shown in Figure 7 and 8.

HALS needs sufficient oxygen supply, which is so different from other artificial organs.[10–19] To overcome this problem, radial flow with porous hepatocytes-immobilizing is effective for HALS.[20–26] From this study, radial flow of medium supply and PAU coated PTFE non-woven fabric scaffold is a good combination for HALS as shown in Figure 4 and Figure 5. The oxygen consumption as well as the glucose consumption rate increased and the hepatocytes were covered with ECM, look-like nature liver tissue even after 7 days

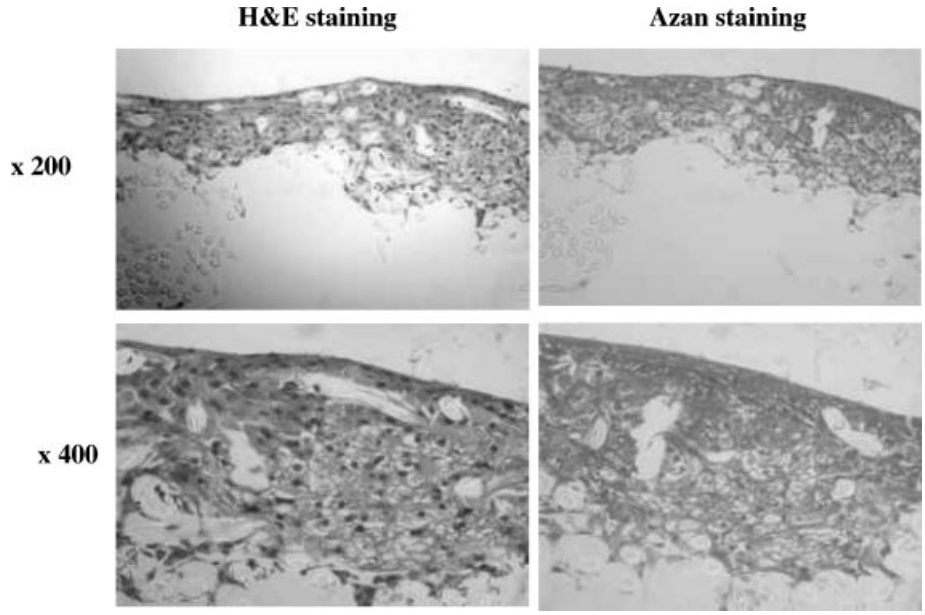

Figure 8.
H&E staining and azan staining of cultured in radial flow bioreactor.

cultured in this projected bioreactor. However, we need to do pre-clinical experiment on animal before any clinical use.

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