Function of a New Internal Bioartificial Liver: An In Vitro Study

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Abstract. The goal of this study was to determine whether a new internal bioartificial liver utilizing porcine hepatocytes can perform detoxification and other metabolic functions. Such a system might aid in treating patients with moderate to severe liver failure and prolong patient survival until a matching organ is found for transplantation. Porcine hepatocytes were attached to a microcarrier and an internal artificial liver was constructed by perfusing the hepatocytes into a polysulfon hollow fiber. The 4 experimental groups were: (a) control group, (b) microcarrier group, (c) hollow fiber group, and (d) internal bioartificial liver group. Viability of hepatocytes, alanine aminotransferase (ALT) and lactate dehydrogenase (LD) activities in the medium, urea production, diazepam transformation, protein synthesis, and glucose-6-phosphatase activity of cells were monitored during a 7-day culture period. Viability of porcine hepatocytes in the internal bioartificial liver group was maintained at >80% during the culture period, and alanine aminotransferase and lactate dehydrogenase activities did not fluctuate significantly. These enzyme activities were significantly lower in the internal bioartificial liver group than in the control or microcarrier groups. Urea production, diazepam transformation, [3H]-leucine incorporation, and glucose-6-phosphatase activity were significantly higher in the internal bioartificial liver group than in the control and hollow fiber groups. These results show that the new internal bioartificial liver produces small amounts of ALT and LD and exhibits detoxification and protein synthetic functions. (received 18 September 2002; accepted 14 February 2003)

Keywords: hepatocyte; internal bioartificial liver; polysulfon, liver functions; liver failure

Introduction

Liver transplantation is one of the most effective treatments for patients with acute liver failure (ALF) [1-3]. The survival rate of ALF has improved from a spontaneous rate of <15% to >70% when a liver is transplanted.[4,5]. However, since donor livers are scarce, only a small number of ALT patients can receive a replacement liver in time.

A bioartificial liver (BAL) may provide necessary liver functions and serve as a temporary liver support [6-9]. Spontaneous recovery of a patient’s native liver by hepatocyte regeneration from hepatic stem cells may be possible when a patient is provided BAL assistance to survive the acute phase of ALF. Extracorporeal BAL has been shown to be effective as a bridge to liver transplantation in some medical centers [8-11], but constructing such a BAL apparatus is very complex.

Although hepatocyte transplantation has been advocated for clinical use because of its simplicity and convenience, immune molecules or immune cells of the host may reject the foreign transplanted cells and kill them. Therefore, immune separation techniques, such as microencapsulation using alginate or polylysine, have been used to avoid immunorejection, but only partial protection has been observed [12,13]. An internal BAL, comprised...
of a semipermeable hollow fiber that contains living hepatocytes, may offer a better alternative than microencapsulated hepatocyte regimens [14-17]. Roger et al [18] demonstrated that treating ALF rats with an implanted BAL, constructed with AN-69 fiber and rat hepatocytes, reduced the mortality rate by 40%.

We recently constructed an internal BAL using a polysulfon hollow fiber and porcine hepatocytes. Before applying it to clinical practice, an in vitro experiment was performed to determine if the internal BAL can provide satisfactory liver functions.

Material and Methods

Animals. Healthy Chinese experimental miniature swine were purchased from the Experimental Center of Beijing Forestry University. The swine were kept individually in a controlled environment (20-25°C, 50-70% humidity) with a 12/12 hr light/dark cycle and were fed a cereal based diet with free access to water for at least a week before the surgical procedure. Access to food was restricted for 12 hr before operation. All animals were treated in accordance with the guidelines of the Affiliated Drum Tower Hospital of the Medical College of Nanjing University.

Hepatocyte preparation. Hepatocytes were isolated from swine by in situ liver perfusion and enzymatic collagenase digestion, as described by Berry and Friend [14], with modifications described by Seglen [15]. Briefly, under ketamine anesthesia (50 mg/kg), median clitoriomy and cannulation of the portal vein were performed. The inferior vena cava was ligated above the renal vein and cannulated close to the heart. The liver was perfused with 3 L of Hanks solution (4°C, pH 7.6) through the portal vein. Then the liver was circularly perfused with 500 ml of 0.5% collagenase solution (Gibco BRL, Merelbeke, Belgium) at a constant rate of 20 ml/min. The softened liver was excised and hepatocytes were separated from the connective tissue by gentle agitation.

The resulting cell suspension was filtered through 50 µm sterile metal mesh. The cells were washed 3 times, suspended in serum-free RPMI 1640 culture medium (Sigma, St Louis, MO) with 0.25 mM glucose, 200 µg/L hydrocortisone, 1 mg/L hepatocyte growth factor, 10 µg/L epidermal growth factor, 20 µg/L nerve growth factor, 100 µg/L insulin, 4 µg/L glucagon, 6.25 mg/L transferrin, 10 mg/L linoleic acid, 2 mmol/L glutamine, 0.5 g/L bovine serum albumin, 3 nmol/L sodium selenate, 0.1 ng/L CuSO₄·5H₂O, 50 pmol/L ZnSO₄·7H₂O, 15 mmol/L HEPES, 200 µg/L cefaperazone, 1 x 10⁵ U/L penicillin, and 100 mg/L streptomycin.

Cell viability was determined by the trypan blue exclusion test. Only suspensions with cell viability of 95% were used. In some groups, Cytodex-3 (Pharmagen, St Louis, MO) was added to attach hepatocytes [16,17]. Cell suspensions were incubated in serum-free RPMI 1640 culture medium overnight at 37°C in 95% air/5% CO₂.

Construction of internal bioartificial liver. Hollow polysulfon fiber was purchased from TECA Corp. (Hong Kong, China). The molecular cutoff was 100 kDa. The internal diameter was 1.0 mm and the wall thickness was 0.1 mm. Before use, the fiber was sterilized, washed with sterile saline, and rinsed in serum-free RPMI 1640 culture medium for 12 hr.

The culture mixture was introduced into the polysulfon fiber (5 x 10⁶ hepatocytes/meter). Ends of the fiber were closed with forceps [18]. The fiber-encased hepatocytes were then cultured in serum-free RPMI 1640 medium at 37 °C in 95% air/5% CO₂.

Experimental design The 4 experimental groups were: (a) control group, (b) microcarrier group, (c) hollow fiber (HF) group, and (d) internal bioartificial liver (BAL) group. In the control group, hepatocytes were directly cultured in non-serum medium. In the microcarrier group, Cytodex-3 was added to hepatocytes (1 g/10⁹ cells). In the HF group, naked hepatocytes were perfused into the hollow fiber. In the internal BAL group, cells attached to Cytodex-3 were perfused into the hollow fiber. The hepatocyte concentrations were adjusted to 1 x 10⁶ cells/ml of medium. On the successive 7 days, samples of the culture medium or cells from each group were collected for study.
**Hepatocyte viability.** Cell viability was tested by the trypan blue exclusion test.

**Release of ALT and LD from hepatocytes.** ALT and LD activities released from hepatocytes into the medium were measured using an automated chemical analyzer (MEGA, Toshiba, Tokyo, Japan).

**Detoxifying functions** (ie, urea production and release by hepatocytes into the medium and diazepam transformation by hepatocytes). Briefly, after porcine hepatocytes were incubation for 24 hr in 10 mM NH₄Cl, urea concentration in the supernatant was measured by a colorimetric assay kit (Randox Laboratories, Antrim, UK) [19].

To assess diazepam transformation, 20 mg/L (w/v) diazepam was added to porcine hepatocytes. After incubation for 24 hr in 5% CO₂ at 37°C, the diazepam concentration in the supernatant was measured using a fluorescence polarization immunoassay (Abbott TDx analyzer, Abbott Park, IL). A blank assay was performed in the absence of cells to adjust for diazepam instability.

**Synthetic functions.** Protein synthesis of hepatocytes was analyzed using the [3H]-leucine incorporation method described by Tong [20,21]. Briefly, 1 µCi/ml [3H]-leucine was added to the medium and hepatocytes were cultured in 5% CO₂ at 37°C for 24 hr. The hepatocytes were washed 3 times with Hanks solution.

Samples were precipitated in 10% trichloroacetic acid (TCA) and 100% ethanol, the precipitates were collected on cellulose filter paper, and radioactivity was counted by liquid scintillation spectrometry (Beckman Instruments, Brea, CA). The radioactivity served as an index of [3H]-leucine incorporated into newly synthesized proteins.

Glucose-6-phosphatase (G-6-Pase), a key enzyme in glycogen synthesis, was also monitored as an index of hepatocyte function. Briefly, hepatocytes were harvested and homogenized in distilled water and G-6-Pase activity (nmol phosphoric acid/10¹⁰ cells) was assayed by measuring the rate of phosphoric acid formation from glucose-6-phosphate [22].

**Statistics.** Assays were performed at least 3 times and the results were expressed as means ± SD. One-way ANOVA with Bonferroni post-hoc analysis was used to determine statistical significance (SPSS software, SPSS Inc, Chicago, IL); p values 0.05 were considered significant.

**Results**

Two Chinese miniature swine were killed for this study; the total hepatocytes procured were 1.4 x 10¹⁰ and 2.1 x 10¹⁰, respectively. Initial viability of the hepatocytes was >95%, and the viability was still >80% after in vitro culture for 7 days. No significant differences were found among the 4 experimental groups of hepatocytes (Table 1).

Release of ALT and LD from hepatocytes into the medium is illustrated in Fig. 1. During the culturing period, ALT and LD activities in the BAL group remained at unfluctuating low levels (ALT, range 8.7 to 21.4 U/L; LD, range 9.8 to 23.4 U/L), which were lower than those in the control and microcarrier groups (p <0.01). The enzyme activities in the HF group were similar to those in the internal BAL group.

Urea production in BAL group was significantly higher than those in the control group and HF group (p <0.01). The urea production in the microcarrier group did not differ from that in the BAL group (p >0.05). (Fig. 2)

The diazepam concentration in hepatocyte supernatants increased continuously in all groups (Fig. 3). In the BAL group, the diazepam concentration was lower than in the control and HF groups (p <0.01). The diazepam concentration in the microcarrier group did not differ significantly from that in the BAL group.

Protein synthesis, measured by [3H]-leucine incorporation, showed a similar trend in the 4 groups, increasing steadily during the period of observation. Incorporation of [3H]-leucine in the BAL group was higher than the HF group (p <0.05). No significant differences were found among the BAL, control, and microcarrier groups (Fig. 4).

G-6-Pase activity in the hepatocytes is shown in Fig. 5. In the first 3 days, the level of G-6-Pase was decreased slightly, and on the fourth to seventh
Table 1. The viability of hepatocytes during the 7-day culture period (n = 3; expressed as %, mean ± SD).

<table>
<thead>
<tr>
<th>Culture period (days)</th>
<th>Control group</th>
<th>HF* group</th>
<th>Microcarrier§ group</th>
<th>BAL† group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97.2±1.4</td>
<td>96.2±1.1</td>
<td>95.8±0.7</td>
<td>97.4±2.0</td>
</tr>
<tr>
<td>1</td>
<td>91.4±4.6</td>
<td>92.6±2.3</td>
<td>89.6±6.5</td>
<td>92.4±4.2</td>
</tr>
<tr>
<td>2</td>
<td>90.8±3.4</td>
<td>93.4±4.7</td>
<td>91.2±4.5</td>
<td>90.4±2.5</td>
</tr>
<tr>
<td>3</td>
<td>87.5±4.3</td>
<td>89.7±3.8</td>
<td>90.3±3.2</td>
<td>91.2±3.1</td>
</tr>
<tr>
<td>4</td>
<td>90.4±3.7</td>
<td>87.4±2.8</td>
<td>88.4±2.9</td>
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</tr>
<tr>
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<td>88.5±4.7</td>
<td>83.4±5.8</td>
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</tr>
<tr>
<td>6</td>
<td>87.6±2.6</td>
<td>85.5±4.1</td>
<td>86.5±1.9</td>
<td>85.3±5.1</td>
</tr>
<tr>
<td>7</td>
<td>84.7±4.0</td>
<td>84.3±2.1</td>
<td>84.3±3.2</td>
<td>83.4±3.3</td>
</tr>
</tbody>
</table>

* HF, hollow fiber group; † BAL, bioartificial liver group.

Fig. 1. Release of ALT and LD by hepatocytes into the supernants of the medium during the 7-day culture period. HF, hollow fiber group; BAL, internal bioartificial liver group.

Fig. 2. Urea concentration in the supernants of the medium during the 7-day culture period.

Fig. 3. Diazepam concentration in the supernants of the medium during the 7-day culture period.
days the level of G-6-Pase tended to increase. In general, the level of G-6-Pase in the BAL group was higher than in the control or HF groups (p <0.01), and did not differ significantly from the microcarrier group.

Discussion

The ideal BAL should function as a normal liver. It should have the potential to support a patient through the worst period of ALF. The internal bioartificial liver is an apparatus based on living hepatocytes and a hollow fiber. The fiber is a semi-permeable membrane that serves as an immune protection barrier and provides a matrix that cells can attach to and grow on. Toxins can pass through the membrane freely and then be detoxified by living hepatocytes. Useful factors secreted by living cells can also pass without difficulty into the blood.

Roger [16] and Gomez [26] constructed an internal BAL based on AN-69 fiber and allogeneic or xenogeneic hepatocytes. The molecular cut-off they chose was 150 kDa and they did not use any microcarriers. The internal BAL they constructed could function for >2 mo following implantation in rat peritoneum. When implanted in rats with ALF, their mortality rate was significantly improved.

The material of the hollow fiber is important. Effectiveness, non-toxicity, economy, and suitability for cell growth are needed. The molecular cut-off of the fiber is a critically important feature of an internal BAL. If it is too large, hepatocytes may not be protected completely; if it is too small, toxins cannot be detoxified and useful factors secreted by hepatocytes cannot enter the body. In our experiment, a polysulfon fiber was chosen, because it has good histocompatibility properties. Since the molecular mass of IgG is about 150 kDa and those of albumin and hepatic growth factor are about 70-80 kDa, we choose a molecular cut-off of 100 kDa.

Living cells are another important factor in internal BAL. It is difficult to get enough normal syngeneic liver cells for ALF patients, so swine hepatocytes were chosen. When separated liver cells were procured, cells membrane may be damaged by enzymes or mechanical factors [23]. Our center previously recommended that 1 to 3 days in culture is needed for cell membrane recovery during the construction of an internal BAL [21]. In this study we constructed the internal BAL after culturing the procured cells for 12 hr and we obtained good results.

Use of a microcarrier helps to improve cell function. Cytodex-3, the most commonly used microcarrier, provides a large surface for cellular attachment, and the attached cells form a microstructure similar to liver. Intercellular messages can be transmitted, so hepatocytes can live and function better. Rosen et al [24] reported that use of a microcarrier improved the mobility and protein
synthesis of epithelial cells. Tang et al [25] found the protein synthesis, amino acid metabolism, and IL-2 secretion were improved by a microcarrier. Therefore, to make the internal BAL function better, we also added a microcarrier (Cytodex-3) to the cell suspension.

In the study, we found ALT and LD activities stayed at low levels in the supernant from the internal BAL group. The reason may be that ALT and LD are large molecules, which passed with difficulty through the fiber membrane. This suggests that, when transplanted into body, the BAL would have little direct effect on serum ALT and LD activities.

Urea production and diazepam transformation were tested to demonstrate detoxification functions of the hepatocytes. The assays of [3H]-leucine incorporation and G-6-Pase activity provided indices of protein biosynthesis of the hepatocytes. In our study, detoxification and synthesis functions in the BAL group did not differ significantly from those in the microcarrier group; the detoxification and synthesis functions in these groups were superior to those in the control and hollow fiber groups. These observations suggest that the polysulfon hollow fiber does not have adverse effects on transmembrane substance exchange and that the microcarrier (Cytodex-3) may improve the cell detoxification and synthesis functions.

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References

22. Burchell A, Hume R, Burchell B. A new micro-technique for the analysis of the human hepatic mesosomal glucose-


