Configuration of a New Bioartificial Liver Support System and In Vitro Evaluation of its Functions

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Abstract. The aim of this study was to configure a new bioartificial liver (BAL) support system and evaluate its functions in vitro. Chinese experimental miniature pig hepatocytes were isolated by an in situ recirculating collagenase perfusion method and were cultured in serum-free medium with restriction attachment and spinner technique to form hepatocyte spheroid suspensions containing 1.0 x 10^{10} hepatocytes. The BAL support system was configured by inoculating the hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor (BIOLIV A3A, Cell Biotech Limited, HK, China). The number and viability of hepatocytes, the levels of alanine aminotransferase (ALT), total bilirubin (TBi), and albumin (ALB) in the circulating hepatocyte suspension and RPMI-1640 medium, and lidocaine metabolism were determined during 6 hr of circulation in the BAL devices. Independent experiments were performed 5 times. There were no significant changes in the number and viability of the hepatocytes during the circulation period. The BAL support system demonstrated substantial albumin synthesis and lidocaine metabolism. The results indicate that the new BAL support system has the ability to perform liver functions and could be used to treat liver failure or provide temporary liver support in patients who are candidates for liver transplantation. (received 16 August 2004; accepted 23 October 2004)

Keywords: bioartificial liver, porcine hepatocyte culture, liver transplantation, lidocaine metabolism

Introduction

Liver failure is associated with high mortality. Currently, orthotopic liver transplantation (OLT) is the most effective way to improve survival in patients with acute liver failure [1]. Because of the shortage of liver donors, long-term immunorejection, and the high cost of transplantation, artificial liver support systems need to be developed. Artificial liver has the potential for providing temporary support for patients with acute hepatic failure and for patients awaiting OLT [2,3]. Bioartificial liver (BAL) devices containing primary hepatocytes have been studied in animal models for several years and some systems are now undergoing clinical trials [1,4-5]. The major problem is how best to configure a BAL support system. To attain enhanced efficacy of liver support, several BAL configurations have been proposed. The long-term maintenance of hepatocyte viability and function is crucial for any BAL support system. Some systems use hepatocyte suspensions, while others use cultured hepatocytes attached to microcarrier beads or multicellular spheroid aggregates [6-10]. In regard to bioreactor design, various bioreactor configurations have been proposed that employ glass plates, hollow fiber membranes, encapsulation in biological matrices, and 3-dimensional carrier materials [11-15].

In this study, porcine hepatocytes were isolated by an in situ recirculating method with collagenase.
perfusion and spinner culture in order to prepare hepatocyte spheroids. A new BAL support system was configured by introducing the hepatocyte spheroids into cell circuit of a BIOLIV A3A hollow fiber bioreactor [16] and the functions of the BAL support system were evaluated in vitro.

Materials and Methods

Animals. Chinese experimental miniature pigs (n = 5, body weight 2.5 to 4.0 kg, male and female) were provided by Beijing Agricultural University and were housed in our research center for 2 wk prior to the experiment. The research protocol complied with Chinese guidelines for humane care of experimental animals. The pigs were allowed free access to water and were fasted for 12 hr before the experiment. One pig was used in each experiment.

Hepatocyte isolation and culture. Collagenase, RPMI-1640 medium, hepatocyte growth factor, nerve growth factor, epidermal growth factor, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were from Gibco BRL Life Technologies, Grand Island, NY, USA. Insulin, glucagon, transferrin, linoleic acid, glutamine, bovine serum albumin, Na2SeO3, CuSO4·5H2O, ZnSO4·7H2O, poly (2-hydroxyethyl methacrylate) (poly-HEMA), penicillin, and streptomycin were from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. Porcine hepatocytes were isolated by an in situ recirculating collagenase perfusion method as previously described [17-19]. The pigs were initially anesthetized with ketamine (50 mg/kg). The abdomen was entered through a median incision. After iv administration of heparin (125 U/kg), the portal vein and infrahepatic vena cava were ligated, respectively, and their proximal portions were cannulated with silicone tubing. The liver was perfused in situ at 37°C for 15 min with 1.5-2.0 L of Ca2+,Mg2+-free Hanks solution. The perfusion was followed by recirculation of 250 ml Hanks solution supplemented with 0.05% (w/v) collagenase at 37°C until the liver surface became grained and the tissue became disrupted. The liver was resected and divided into several parts. The tissue was washed and weighed, after which it was put into a sterile bottle and digested with 0.05% (w/v) collagenase at 37°C for 15 min. Following the enzymatic digestion, the hepatocyte suspension was passed through a 120 µm stainless steel sieve. It was washed with Hanks solution immediately and hepatocytes were isolated by centrifugation (1000 rpm, 5 min) 3 times at 4°C. Cell viability was assayed by the trypan blue exclusion test.

Hepatocytes were inoculated at a density of 5 x107/ml in serum-free RPMI-1640 medium supplemented with 200 µg/L hydrocortisone, 1 mg/L hepatocyte growth factor, 10 µg/L nerve growth factor, 20 µg/L epidermal 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 Na2SeO3, 0.1 µmol/L CuSO4·5H2O, 50 pmol/L ZnSO4·7H2O, 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 200 mg/L cefoperazone sodium, 100,000 U/L penicillin, and 100 mg/L streptomycin. To inhibit cell attachment and to induce the formation of cell spheroids in suspension, 250 ml bottles were coated with poly-HEMA. About 100 ml of hepatocyte suspension was placed in each bottle. The bottles were placed in an incubator (5% CO2, 37°C) for 20 hr and slowly rotated (12 revolutions/hr). The hepatocytes were counted and their viability was determined by the trypan blue exclusion test. The morphology of the hepatocytes was inspected with an inverse microscope.

Configuration of the BAL support system. We developed a BAL support system that contained a hollow fiber cartridge (BIOLIV A3A, Cell Biotech Ltd, Hong Kong, China) with a nominal molecular weight cut-off of 70 kDa, a pore size of 200 nm, and a surface area 0.06 m2. The BAL support system is diagrammed in Fig. 1. The total cell circuit volume (including the bioreactor) was 250 ml. Hepatocyte spheroids in serum-free RPMI-1640 medium containing 1.0 x 1010 primary porcine hepatocytes were infused into the outer space of of the hollow fibers and the medium was circulated at 20 ml/min with continuous O2 input (2 L/min). The bioreactor was kept at 37.5°C in an incubator. The RPMI-1640 medium flowed through the lumen of the hollow fibers at a rate of 30 ml/min for 6 hr.
Fig. 1. Schematic diagram of the BAL support system. The BAL system has two circuits. Pump 1 circulates an oxygenated suspension of porcine hepatocyte spheroids in the outer space of the hollow fiber bioreactor (BIOLIV A3A). Pump 2 circulates RPMI-1640 medium in the inner space (lumen) of the hollow fiber bioreactor. The entire BAL support system is housed in an incubator at 37°C.

Fig. 2. Total numbers of porcine hepatocytes (x 10^10) in the cell circuit of the BAL support system, based on cell counts at the start of circulation and 2, 4, and 6 hr thereafter. The circles with error bars show the means ± SD of results from 5 independent experiments.

Fig. 3. Viability of porcine hepatocytes (%) in the cell circuit of the BAL support system, based on trypan blue exclusion tests at the start of circulation and 2, 4, and 6 hr thereafter. The circles with error bars show the means ± SD of results from 5 independent experiments.
Fig. 4. Time-course of alanine aminotransferase activity (ALT, U/L) in samples from the two circuits of the BAL support system at the start of circulation and 2, 4, and 6 hr thereafter, based on 5 independent experiments. The triangles with error bars show the ALT activity (mean ± SD) in samples of the circulating suspension of hepatocyte spheroids; the circles with error bars show the ALT activity in the samples of RPMI-1640 medium. *p <0.05 for ALT activity in the hepatocyte suspensions vs the corresponding RPMI-1640 samples.

Fig. 5. Time-course of total bilirubin concentration (TBi, mmol/L) in the two circuits of the BAL support system at the start of circulation and 2, 4, and 6 hr thereafter, based on 5 independent experiments. The triangles with error bars show the TBi levels (mean ± SD) in the circulating suspension of hepatocyte spheroids; the circles with error bars show the TBi levels in the samples of RPMI-1640 medium. *p <0.05 for TBi concentration in hepatocyte suspensions vs the corresponding RPMI-1640 samples.

Fig. 6. Time-course of albumin concentration (ALB, g/L) in the two circuits of the BAL support system at the start of circulation and 2, 4, and 6 hr thereafter, based on 5 independent experiments. The triangles with error bars show the ALB levels (mean ± SD) in the circulating suspension of hepatocyte spheroids; the circles with error bars show the ALB levels in the samples of RPMI-1640 medium. *p <0.05 for ALB concentration in hepatocyte suspensions vs the corresponding RPMI-1640 samples.
Cell number and viability assays. The number and % viability of hepatocytes in the cell circuit pre-circulation and at 2, 4, and 6 hr of circulation were assayed by cell counting and trypan blue exclusion.

Biochemical analyses. Independent experiments were performed 5 times with the BAL devices. Samples of the hepatocyte suspension and RMPI-1640 medium were obtained at 2 hr intervals during 6 hr of circulation. Changes of alanine aminotransferase (ALT), total bilirubin (TBi), and albumin (ALB) levels in the circulating hepatocytes and the medium were determined with an automatic biochemical analyzer (MEGA, Toshiba, Japan).

Lidocaine metabolism test. The experimental and control groups (no cells) were set up. Lidocaine (40 mg/L) was added to RPMI-1640 medium when the circulation began. Samples (0.5 ml) of RPMI-1640 medium was obtained after 15 min, 1, 2, 4, and 6 hr of circulation. Monoethylgycylnexylidide (MEGX) was determined by a fluorescence polarization immunoassay (TDX analyzer, Abbott Laboratories, North Chicago, IL, USA).

Statistical analysis. Results were expressed as means ± SD; statistical differences were evaluated by ANOVA and paired-sample t tests. The threshold for statistical significance was p <0.05.

Results

Number and viability of hepatocytes. The yield of isolated hepatocytes from a single porcine liver averaged 1.52 x 10^{10} ± 0.25 x10^{10} (range = 1.0 x 10^{10} to 2.5 x 10^{10}). At the start of the experiments, hepatocyte viability averaged 97%. Hepatocyte spheroids formed after incubation for 20 hr in the serum-free medium with restricted attachment and spinner culture. The average viability of hepatocytes was maintained at 95% throughout the experiments. As shown in Figs. 2 and 3, there were no significant changes in the number or viability of porcine hepatocytes during the 6 hr of circulation.

ALT, TBi, and ALB levels in hepatocyte suspensions and RPMI-1640 medium during circulation. ALT, TBi, ALB levels in hepatocyte suspensions and RPMI-1640 medium during circulation experiments are shown in Figs. 4 to 6. In the hepatocyte suspensions, there were no significant changes of ALT activity during 6 hr of circulation, whereas the TBi and ALB levels increased gradually (p <0.05). In the RPMI-1640 medium, the pre-circulation levels of ALT, TBi and ALB were lower than in hepatocyte suspensions (p <0.05). During 6 hr of circulation, there was no significant change of ALT activity, whereas the TBi and ALB levels gradually increased (p <0.05). At 2, 4, and 6 hr of circulation, there were no significant differences of TBi levels in RPMI-1640 medium vs the hepatocyte suspensions, but the ALB concentration was lower in the RPMI-1640 medium than in hepatocyte suspensions at all times (p <0.05).

Lidocaine metabolism test. In the samples of hepatocyte suspensions, MEXG levels averaged 32.3 ± 3.7, 45.7 ± 3.9, 39.2 ± 5.3, 33.2 ± 4.2, and 21.3 ± 3.8 µg/L after circulation for 15 min, 1, 2, 4, and 6 hr, respectively. The MEXG level peaked at 1 hr and then declined gradually. MEXG could not be detected in the corresponding samples of RPMI-1640 medium at any time.

Discussion

Treatment of acute hepatic failure is a formidable clinical challenge. In China, an area with high incidence of viral hepatitis, the morbidity and mortality from acute and chronic hepatic failure are very high. At present, OLT is the most effective therapy, but the patients’ serious condition, the scarcity of donor organs, and high costs limit the general availability of OLT. An artificial liver support system could serve as a bridging technique until a donor organ becomes available [2,3]. Furthermore, it might allow some patients with acute hepatic failure to recover their hepatic function.

Preliminary clinical trials indicate that BAL is a promising therapy for acute hepatic failure and a bridge to liver transplantation [1]. An ideal BAL system would provide all of the hepatic functions. BAL systems differ from non-biologic artificial liver devices in the synthesis of essential metabolites and
the selective removal of toxic substances, which are carried out by the cultured hepatocytes. The choice of hepatocyte components, the methods of isolation and culture, and the number of hepatocytes are key points in the configuration of BAL [1].

In theory, human hepatocytes are the optimal choice, but the scarcity of donor livers restricts their use in BAL. Since porcine hepatocytes are similar to human hepatocytes in regard to morphology and biological functions, they can serve as a heterogeneous resource for BAL [17,18]. In recent years, a BAL device based on porcine hepatocytes was admitted into clinical trials by the Food and Drug Administration in the USA.

Configuration of BAL needs a large number of hepatocytes with high viability. In this study, porcine hepatocytes were isolated in situ by recirculating collagenase perfusion [19]. The method consumed less collagenase and yielded better cell viability (ie, 97%) than other methods. The number of hepatocytes is also an important parameter of BAL, since 10-30% of hepatocytes of the normal human liver are needed to maintain normal hepatic functions [20]. Matsmura et al [21] proposed that a BAL requires 150 to 450 g of liver. In clinical studies of BAL, the number of cultured hepatocytes ranges from $10^9$ to $10^{10}$ [7]. The BAL device that we configured in the present study contained at least $1.0 \times 10^{10}$ hepatocytes. Large numbers of hepatocytes must be cultured at high density because of the limitation of BAL volume.

It has been reported that cell-cell interaction has an important role in maintaining the viability and functions of hepatocytes [22]. In the present study, hepatocytes were incubated in serum-free medium and poly-HEMA-coated bottles by a continuous rotational method in order to restrict their attachment to the wall and promote the formation of hepatocyte spheroids. This method not only facilitates cell-cell interaction and maintains cell functions, but it also solves the requirement of high density culture in BAL [22]. It reduces the possibility of immune reactions by using serum-free medium with hormones and various growth factors [7]. The present study demonstrated the formation of hepatocyte spheroids by spinner culture with 97% viability, compared to the 75 to 85% viability of hepatocytes that has previously been obtained by a common culture technique [16].

The design of the bioreactor is another key point in BAL. The ideal bioreactor should provide a good environment for hepatocyte growth and metabolism as well as for the efficient exchange of substances. At present, the most common device is a hollow fiber bioreactor with many small hollow fibers made from semipermeable membranes. The device has two independent compartments that are separated by the hollow fiber semipermeable membranes. The intratubular space is used to perfuse blood, while the extratubular space is used to culture hepatocytes. The patient’s blood or plasma flows through the bioreactor, exchanging substances with hepatocytes through the semipermeable membranes. The membranes also provide immuno-isolation. For optimal substance exchange, hollow fiber chambers with high molecular weight cut-off (MWCO) membranes are best [23]. But for optimal immuno-isolation, hollow fiber chambers with low MWCO membranes are best. In view of the molecular weight of albumin (68 kDa), a hollow fiber membrane with 200 nm pore size and a MWCO of 70 kDa was chosen for the present study. The membrane allowed passage of some relatively small molecules, such as albumin, but restricted the passage of lymphocytes and higher molecular weight proteins.

The faults of hollow fiber bioreactors include maldistribution of hepatocytes, poor adherence of hepatocytes and the formation of large aggregates of hepatocytes during circulation. Such problems affect the functions of the hepatocytes, block the membrane pores, and impair substance exchange and oxygen transport. Hay et al [24] reported that the oxygen supply was inadequate in a polysulfone hollow fiber bioreactor (Hepatix Co., La Jolla, CA, USA). Therefore, in our new BAL system, we devised a cell circuit with continuous $O_2$ input. Although number of hepatocytes in a bioreactor was at least $10^{10}$ and the density of hepatocytes attained $5 \times 10^7$/ml, the hepatocytes received sufficient nutrients and oxygen supply. As a result, the hepatocyte viability remained high throughout the 6 hr of circulation.

Our results showed that the TBi and ALB levels in the hepatocyte suspensions and in the RPMI-1640 medium increased during 6 hr of circulation.
There were no significant differences of TBi levels in RPMI-1640 medium vs hepatocyte suspensions after 2 hr of circulation. The ALB level in the RPMI-1640 samples was lower than in the hepatocyte suspensions at all periods of circulation. The ALB levels in RPMI-1640 medium and hepatocyte suspension averaged 2.8 g/L and 3.8 g/L, respectively, after 6 hr of circulation. During the 6 hr of circulation, there were no significant changes of ALT levels in RPMI-1640 medium or hepatocyte suspensions. The level of ALT was lower in RPMI-1640 medium than in hepatocyte suspensions at all times. Bilirubin and albumin could readily cross the semi-permeable membrane because of their low molecular weights. The results indicate that albumin synthesized by hepatocytes in our BAL support system can cross into into the circulating stream in the intratubular space of the hollow fibers.

The biotransformation functions of the hepatic cytochrome P450 (CYP) system may determine whether patients recover from liver failure. Therefore it is important to evaluate the biotransformation functions of BAL [25]. Lidocaine is quickly metabolized mainly in liver. About 90% of lidocaine is transformed into monoethylglycinexylidide (MEGX) and glycinexylidide (GX) by CYP-mediated, hepatocyte-dependent dealklation reactions. The blood MEGX level of healthy persons increases rapidly during the period from 0 to 15 min after iv injection of lidocaine. The MEGX level reaches a peak at 15 min and stays elevated from 15 to 60 min [26]. In contrast, in patients with hepatic impairment, blood MEGX level increases slowly and reaches a peak at about 4 hr after iv injection of lidocaine [27]. The lidocaine metabolism test has been used to estimate the biotransformation functions of BAL [25]. Iwata et al [25] reported that a hollow-fiber BAL device maintained lidocaine metabolism during a 10-day observation period. In the present study, the MEGX level in the new BAL device averaged 32.3 ± 3.7 µg/L after 15 min of circulation and reached its peak (45.7 ± 3.9 µg/L) after 1 hr of circulation. Thereafter, the MEGX level declined gradually. These findings indicate that the BAL displays the biotransformation function of the hepatocyte cytochrome P450 system and could partially replace the hepatic functions of detoxification and metabolism. Such measurements of lidocaine metabolism in a BAL support system have not previously been reported.

In conclusion, we developed a new hollow fiber BAL support system with two circuits. Porcine hepatocyte spheroids are infused and oxygen flows into the cell circuit. In vitro studies showed that the BAL device has an ability to support liver functions and appears to have potential advantages for clinical use in patients with liver failure.

Acknowledgments

This study was supported by a grant from the Health Department of Jiangsu Province, People’s Republic of China.

References

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