Effect of Low Temperature Preservation and Cell Density on Metabolic Function in a Bioartificial Liver

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Abstract Difficulties associated with bioartificial liver (BAL) preservation limit not only the commercialization of BAL, but also its clinical trials. In this study, the possibility of cold preservation of BAL cartridges containing porcine hepatocytes was examined at 4 °C. In an *in vitro* perfusion culture system, BAL cartridges maintained cytochrome P450 metabolic function for at least 50 days. However, all BAL cartridges completely lost their ammonia eliminating ability when stored at 4°C. We also studied the effect of cell density on the maintenance of BAL liver function in a highly differentiated and healthy state. As expected, BALs containing a larger number of hepatocytes demonstrated higher metabolic functions. When metabolic functions were compared per gram of hepatocytes, no large differences were observed between devices containing different densities of hepatocytes. Decreased cell density did not successfully prolong BAL function. The viability and function of isolated hepatocytes highly depend on the culture conditions, such as cell density, substrata, culture media, and additives to the culture media. Perfusion culture of BAL cartridges at 4°C gave a promosing result with respect to the maintenance of P450 activity. However, as indicated by the rapid loss of ammonia metabolic activity, many factors still remain to be optimized for preservation of BAL keeping high metabolic functions for a longer time.

Keywords: cold preservation, bioartificial liver, lidocaine, ammonia, cell density

INTRODUCTION

The liver performs many important metabolic functions and is the only internal organ that has the capacity to regenerate itself with new healthy tissues. Loss of liver cell functions may result in the disruption of many essential metabolic functions, which could lead to death. At present, the use of BAL system as a temporary bridge to liver transplantation is one promising approach to treat patients with severe liver failure due to the shortage in specific organ donors. BAL is a liver assist device that contained entrapped living hepatocytes or immortalized cells derived from hepatocytes in a cartridge [1-5]. These devices were developed based on the assistance of the metabolic functions of the liver. Many groups have tried to maintain the functions and viability of the hepatocytes in BAL devices for a long term.

Conventional artificial organs and various medical devices made of non-living materials can be stored for a long time, and thus, can be supplied to patients when needed. On the contrary, it has been pointed out that bioartificial organs containing living cells are hard to store and that manufacturers or distributors are faced with the problem of maintaining large stocks in order to ensure a steady supply. Thus, the preservation is one of major obstacles to be overcome before commercialization. Since hepatocytes rapidly lose many liver-specific functions including their ability to detoxify, which is attributed to the actions of cytochrome P450 enzymes and cytosolic metabolic functions in *in vitro* culture, their cryopreservation seems to be difficult employing conventional methods, although such methods have been successfully used to cryopreserve various other cells [6-8]. This inability to adequately store BALs inhibits commercialization and clinical trials. Therefore, much effort has been put into preserving BAL cartridges whilst preventing the loss of differentiated hepatocytes functionality. Pahernik *et al.* [9,10] investigated the effect of low temperature storage time at 4°C on the ultrastructure of cells and their functional capacities using three different protective solutions and reported that porcine hepatocytes maintained the cytochrome P450 dependent activity for several days, when stored at 4°C in University of Wisconsin solution. However, several days preservation is not sufficient.

In this study, we examined the possibility of low temperature BAL cartridge preservation at 4°C, and investigated the effects of cell density on the maintenance of

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hepatocytes in a highly differentiated. BAL metabolic functions after periods of preservation were evaluated using a lidocaine loading test and the results obtained quantitatively represented as clearance values.

MATERIALS AND METHODS

Preparations of BAL Cartridges and *In Vitro* Circulation

BAL cartridges were prepared using porcine hepatocytes using the method previously reported paper [11]. Briefly, porcine hepatocytes were isolated from pigs, of approximately 12-kg body weight, using the collagenase digestion method [12,13]. After digestion, the liver capsule was incised on all lobes and hepatocytes were released into calcium- and magnesium-free Hanks' balanced salt solution (HBSS) at 4°C. The cell suspension was gently filtered through a 100 μm nylon mesh opening into 1,600 mL of iced HBSS. The filtrate was divided into 8 polypropylene test tubes with 225 mL and then centrifuged at 50 g for 3 min. This washing procedure was repeated 3 times to remove collagenase. Cell count and viability were assessed using the trypan blue dye exclusion test using 0.4% trypan blue dye in phosphate buffered (PBS) and a hemocytometer.

Hollow fiber cartridges (FB-50F, Nipro, Osaka, Japan), which had been used clinically as a hemo-dialyzers for child patients, were used to prepare BAL cartridges. The hollow fibers in the cartridge were made of cellulose triacetate with a nominal cut- off molecular weight of 70 kDa, and their inner and outer diameter were 200 and 230 μm, respectively. The total volume of the inner space of the hollow fibers was 35 mL. The volume of the shell space between the hollow fibers and the cartridge housing was 50 ml. Thirty five mL of the suspension was immediately inoculated through the arterial end of the cartridge into the inner space of the hollow fibers. Both ends of the hollow fiber cartridge were then closed using two plastic caps.

In Vitro Circulation of BAL Cartridges

The bioreactor consisted of a glass bottle reservoir of 500 mL, a roller pump (BP-02, JMS, Hiroshima, Japan) a dense membrane oxygenator (total fiber surface area: 0.4 m^2, used for extracorporeal membrane oxygenation for a child patient, MENOX AL-2000, KURARAY, Osaka, Japan) and the BAL cartridge connected by PVC tubing. The total volume of the circuit was 200 mL except the reservoir. The culture medium, consisted of 100 mL of the human whole blood and 400 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5 μg of EGF, 2.5 μg of bFGF, 1.85 g bicarbonate, 1,250 unit heparin, 20 unit insulin (Humulin R, Lilly Japan, Kobe, Japan), 5×10^4 units of penicillin, and 0.05 g of streptomycin, was perfused in a closed circuit through the lumen of the BAL cartridge. The perfusate in the reservoir was well mixed with a magnetically

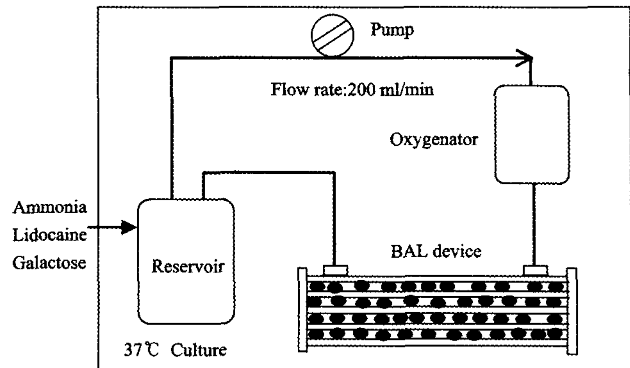

Fig. 1. Schematic representation of BAL device and the *in vitro* circulating system used for the evaluation of its functions.

coupled stirrer bar. The shell portion of the cartridge was perfused at 200 mL/min, and oxygenated using a 95% air and 5% carbon dioxide gas mixture. Five hundred units of heparin were administered by bolus every 24 h. For long term cultivation, the perfusate was replaced twice a week with fresh dilute human blood.

For low temperature preservation, the BAL cartridges were prepared by inoculating hepatocyte suspension containing 1.0×10^8 cells/mL. The closed circuit was then incubated at 37°C for 24 h and then at 4°C until the functions of BAL were lost, in a flow culturing system (Hollow fiber reactor HFR-1000, EYELA, Tokyo, Japan). BAL system to evaluate the function of lidocaine and ammonia was kept 37°C before 3 h of test and then back at 4°C after initial bolus loading test.

To determine the effect of cell density, 35 mL of hepatocyte suspension at 1×10^8, 3.3×10^7 or 1.7×10^7 cells/mL in HBSS were inoculated into hollow fiber cartridges, which were then placed in the closed circuit and cultured at 37°C as shown in Fig. 1. The effects of cell density on the function of BAL were evaluated using lidocaine, ammonia, and galactose.

Chemical Loading Test

The test was performed using lidocaine, ammonia, and galactose as chemicals as described previously paper [14]. Briefly, for an initial bolus loading, the lidocaine and ammonium hydrochloride in PBS solution were injected by bolus to the perfusate at a concentration of 10 μg/mL and 1,700 μg/dL, respectively. For lidocaine, 1 mL of the perfusate was collected at 1, 2, 3, 4, 5, 7, 9, 10, 15, 30, 45, 60, 75, 90, 105, 120, and 180 min after the initial bolus administration. For ammonia, 2 mL samples were collected at 1, 15, 30, 60, 90, 120, and 180 min after the initial bolus administration. The supernatant was collected after centrifugation at 1,800 g for 7 min and stored at –20°C prior to analysis. For galactose, the drug in PBS solution was bolusly added to the perfusate with concentration of 6.25 mg/mL, and 1 mL of the perfusate was collected every 3 h for 48 h.

The levels of lidocaine, ammonia, and galactose in the

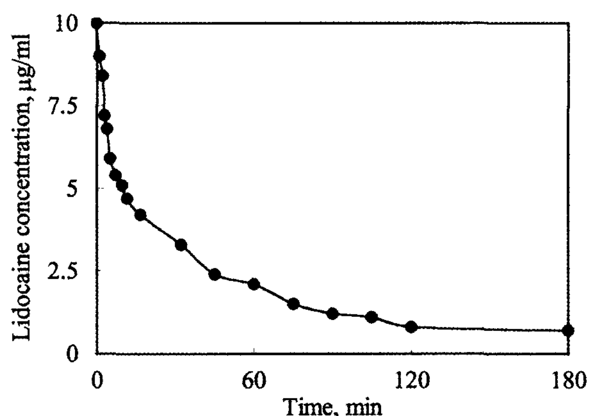

Fig. 2. Lidocaine concentration change with time for *in vitro* BAL system at day 5.

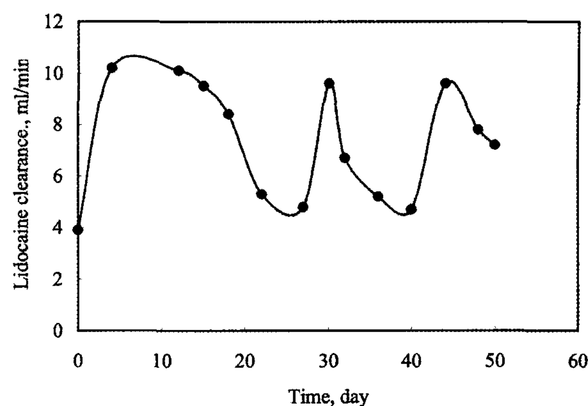

Fig. 3. Change of lidocaine CL of BAL preserved at 4°C.

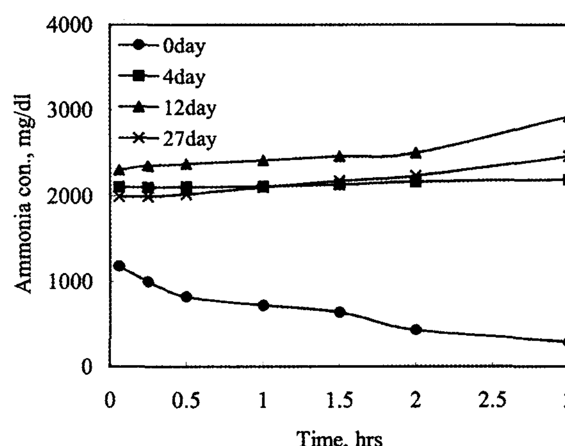

Fig. 4. Ammonia concentration change in the reservoir after 1,700 μg/dL loading of ammonia. BAL was preserved at 4°C for 0, 4, 12, and 27 days.

perfusate samples were determined using a TDX analyzer (Dainabot, Tokyo, Japan), an F-kit for ammonia analysis in food (Boehringer Mannheim, Germany), and an F-kit for Lactose/D-Galactose (Boehringer Mannheim, Germany), respectively. The metabolic efficacy of the BAL device was expressed using the clearance values of the loaded chemicals.

RESULTS

Low Temperature Preservation of BAL

The cytochrome P450 system in hepatocytes includes the most important detoxification enzymes, and hence, P450 activity should be maintained by BAL cartridge. Moreover, lidocaine is known to be metabolized by oxidative N-demethylation to monoethylglycinexylidine (MEGX) in the cytochrome P450 system, and that the ammonia concentration in the blood of patients with severe liver failure increases with time. BAL cartridge must effectively remove this ammonia from the blood. Lidocaine and ammonia loading tests were thus performed to determine the metabolic capacity of BAL cartridge after low temperature preservation for set periods of time. Fig. 2 shows a representative result of lidocaine concentration change in the perfusate. The data points of lidocaine were obtained to calculate CLs than those of MEGX due to the same ratio of decreasing of lidocaine concentration to the production of MEGX (data is not shown). This figure demonstrates the time course of the concentration changes observed for lidocaine loading tests performed after 5 days of low-temperature storage. Apparently, the lidocaine was effectively eliminated from the perfusate within several hours. The concentration decay curves in Fig. 2 were analyzed using a physiological pharmacokinetic model [14] and the metabolic efficiency was expressed using the clearance (CL). CL values calculated from the changes of lidocaine concentration are shown in Fig. 3, which also shows CL. Here, CL (mL/min) is the clearance of BAL device that is defined as a volume of a perfusate from which entire drug removed in unit time in BAL. Figure shows that BAL cartridge maintained lidocaine metabolic function over at least 50 days of observation.

Examples of ammonia concentration changes in the perfusate are shown in Fig. 4. Apparently, ammonia was effectively removed from the perfusate at day 0. However, BAL cartridges completely lost its ammonia eliminating ability when stored at 4°C. Ammonia concentration in the perfusate did not decrease with time, but increased slightly as shown in Fig. 4.

Effect of Cell Density

Hepatocyte suspension of different cell densities were inoculated into the hollow fiber lumen to study the effect of cell density on the maintenance of metabolic functions. Fig 5(A) shows changes of the lidocaine CL value, expressed in mL/min, with culture time, while Fig. 5 (B) shows the CL value per gram of hepatocytes. Ammonia CL values are shown in Fig. 6(A) and (B). The galactose-

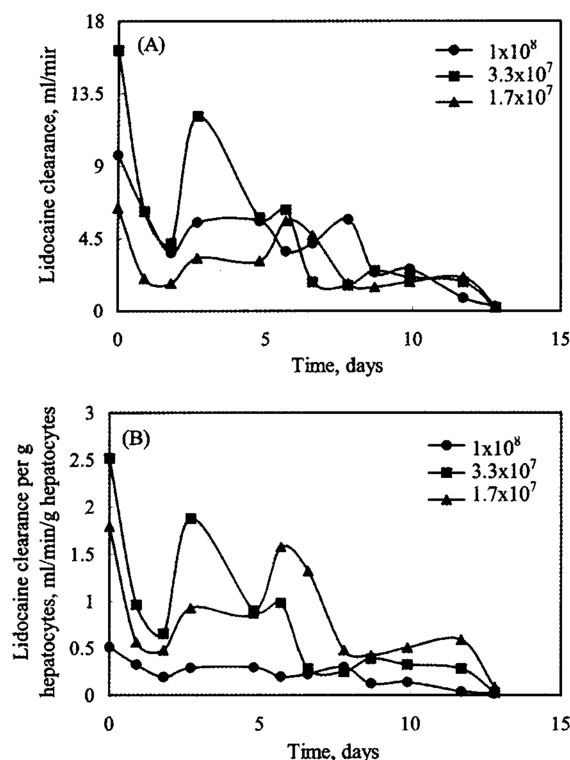

Fig. 5. Change of lidocaine clearance of BAL prepared by inoculation of hepatocytes at different cell densities. (A) Lidocaine clearance, (B) Lidocaine clearance per gram of hepatocytes.

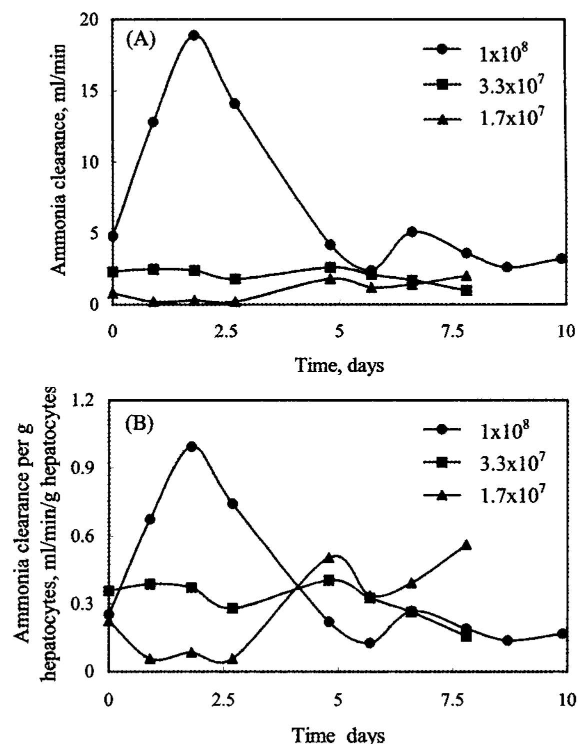

Fig. 6. Change of ammonia clearance of BAL prepared by inoculation of hepatocytes at different cell densities. (A) Ammonia clearance, (B) Ammonia clearance per gram of hepatocytes.

loading test was conducted at a concentration similar to that used for clinical tests. Galactose elimination capacity (GEC) [15], instead of the clearance, was determined from the slope of the galactose concentration decay curve and used as a measure of the galactose metabolic activity of the BAL, as described previously [14]. Here, GEC (mg/min) means maximum elimination rate in BAL. The observed GEC values are plotted against the perfusion time in Fig. 7(A) and (B).

As expected, the BAL containing the larger number of hepatocytes showed a higher level of metabolic functions. When metabolic functions were compared per gram of hepatocytes, no significant difference was observed between the devices containing different densities of hepatocytes. Moreover, metabolic ability changes versus culture period was differed slightly from device to device. However, the culture period at which all of these metabolic functions decreased to less than 30% of the maximum value was 7-10 days. Cell density reductions did not prolong the BAL function period.

Metabolic activity per gram of hepatocytes depended on the kind of chemicals loaded. Hepatocytes in BAL cartridge prepared using low cell density suspension, such as, $3.3\text{-}1.7\times10^7$ cells/mL, demonstrated higher lidocaine and galactose elimination abilities than those in BAL cartridges prepared using suspension of 1×10^8 cells/mL. However, no significant difference was observed between these cartridges in terms of cell densities. All of BAL cartridges gradually lost lidocaine and galactose metabolic ability after 7 days of culture. In the case of the ammonia-loading test, hepatocytes in BAL cartridge prepared using 1×10^8 cells/mL suspension showed the highest metabolic activity over the initial 3 days of culture. Hepatocytes in the cartridge containing prepared using a suspension of 1.7×10^7 cells/mL gradually gained in metabolic activity. Although some dependence between metabolic activity and the maintenance of cell density was observed, a decrease in cell density did not necessarily produce any beneficial effect in terms of the long term preservation of BAL cartridges.

DISCUSSION

Hepatocytes were inoculated into a hollow fiber cartridge with densities as high as possible to prepare BAL with efficient metabolic functions [11]. BALs prepared effectively removed loaded lidocaine, ammonia, and galactose, but gradually lost their metabolic functionality with time. For instance, metabolic functions decreased to less than 30% of their maximum values after 7 days of culture. A major problem associated with the maintenance of cell viability and functionality may be limited mass transfer, especially oxygen to the cells. When hepa-

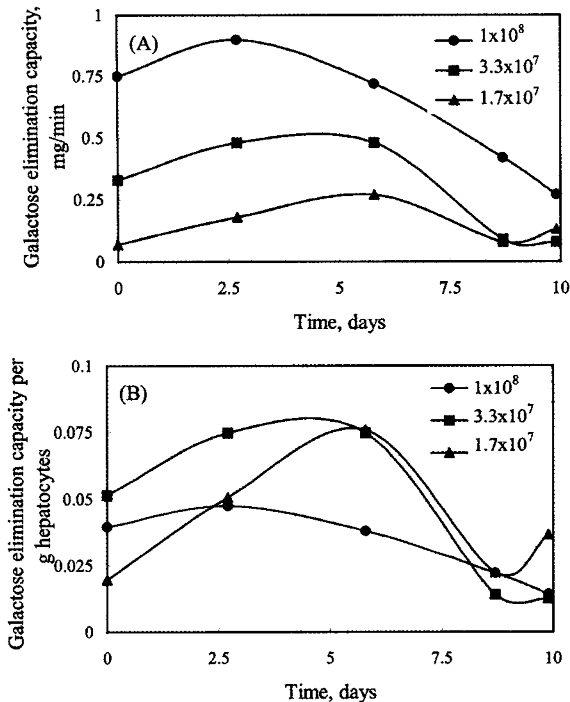

Fig. 7. Change of galactose elimination capacity of BAL prepared by inoculation of hepatocytes at different cell densities. (A) Galactose elimination capacity, (B) Galactose elimination capacity per gram of hepatocytes.

tocytes are inoculated into hollow fibers at a high density, the cells compete against each other for oxygen and nutrients supplied through the hollow fiber membrane from the perfusate. In addition to this effect, a high cell density disturbs the diffusions of these nutrient. These may be the limiting factors that prevent the long-term preservation of BAL cartridges.

Cryopreservation has been suggested to store a pool of hepatocytes for the preparation of BALs, but the technique usually results in the substantial loss of cells, especially of their liver specific functions. Pahernik *et al.* [9, 10] investigated the effects of low temperature storage at 4°C on the ultrastructure of cells and their functional capacities using three different protective solutions and reported that porcine hepatocytes maintained cytochrome P450-dependent activity for several days, when stored at 4°C in a University of Wisconsin solution. Our BAL maintained its lidocaine metabolic function for at least 50 days when stored at 4°C. Apparently, then a great improvement realized during our study, but the same BAL completely lost its ammonia metabolic capacity when exposed to 4°C from initial test. Pahernik *et al.* did not examine changes of the ammonia metabolizing ability of their hepatocytes after storage at 4°C. Many studies have been performed on liver injury due to low-temperature preservation in connection with primary non-functioning of transplanted liver, but, to our knowledge, no study has found that low-temperature preservation drastically impaired the ammonia metabolizing activity of the liver. Our finding may give an important insight into the primary non-functioning of the transplanted liver.

Hepatocytes are readily recovered *in vivo* even after injury induced by chemicals or viruses, as does the liver after surgical removal, and they have a tremendous capacity to replicate [16,17]. Bovine and porcine hepatocytes can proliferate many times on tissue culture disks, when isolated from a fetal or neonatal donor, and kept under well-controlled conditions [18]. We expected, these facts, that hepatocytes inoculated into BAL cartridge at low density, would be well supplied with oxygen and nutrients. As mentioned above, however, the observed results were not as good as had been anticipated, although the metabolic functions of BAL with low cell densities increased with time and were maintained for a long time.

The viability and the functions of isolated hepatocytes depend greatly on the culture conditions, such as, the cell density, the substrate, the culture media, and the types of additives in the culture media. Perfusion culture of BAL cartridge at 4°C gave a promising result with respect to the maintenance of P450 activity. However, as indicated by the rapid loss of ammonia metabolizing activity, many factors remain to be optimized for preservation of BALs over longer period of time.

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