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Determination of Optimum Aggregates of Porcine Hepatocytes As a Cell Source of a Bioartificial Liver

LEE, DOO-HOON[1], JI-HYUN LEE[2], JEONG-EUN CHOI[3], YOUNG-JIN KIM[3], SUNG-KOO KIM[2], AND JUNG-KEUG PARK[1]*

[1]*Department of Chemical and Biochemical Engineering, Dongguk University, Seoul 100-715, Korea*
[2]*Division of Food and Biotechnology, Pukyong National University, Pusan 608-737, Korea*
[3]*Lifecord Biomedical Research Center, Lifecord Inc., Suwon 442-721, Korea*

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Abstract Large quantities of porcine hepatocyte aggregates with various degrees of aggregation (DA) could be obtained by controlling the suspension periods (0, 9, 24, and 48 h), and by entrapping the hepatocyte aggregates in model materials of encapsulation such as Ca-alginate and type-I collagen gels. The effects of DA on liver-specific functions of hepatocytes were evaluated in order to obtain optimum DA for the cell source of bioartificial liver (BAL) systems. Irregular rugged aggregates (size 75±28 μm) formed by 24 h of suspension culturing showed peak viability and hepatic functions such as ammonia removal and albumin secretion in the two types of entrapment systems, thus offering themselves as a stable cell source of a BAL system for hepatic functions and scale-up.

Key words: Bioartificial liver, degree of aggregation, hepatocyte spheroid, porcine hepatocyte

Bioartificial liver (BAL) is an extracorporeal liver support system, which contains biological components such as hepatocytes. The BAL is to serve as a bridge to provide patients with the extension of survival time until a donor organ becomes available for transplantation or until their own liver can be regenerated [4]. The performance of a BAL depends on the functional activities of the hepatocytes immobilized in the system [3]. One of the major factors influencing hepatic functions of cultured hepatocytes is direct cell-cell interactions between hepatocytes [8], which increase as cell aggregation progresses. However, little research has been done on the effect of degree of aggregation (DA) on the maintenance of the differentiated functions of the hepatocytes. Furthermore, in spite of the lack of cell-cell interaction at single cells and mass transfer limitation at complete spheroids, most BAL systems using encapsulated hepatocytes utilize initial dispersed single cells [2] or fully aggregated spheroids of 200 to 300 mm diameter [6, 15]. Therefore, it is a prerequisite for the development of a high performance BAL system to find optimum hepatocyte aggregation under encapsulation conditions. Patricia and Saltzman [13] used small aggregates, having only 4–6 hepatocytes each, but they could not evaluate the effects in detail. Surapaneni *et al.* [16] examined a broad range of aggregate sizes, however, their aggregation method using a tissue culture flask generally required a large surface area to form large quantities of aggregates for the BAL system.

In this study, large quantities of porcine hepatocyte aggregates with various DA were prepared by controlling the suspension periods of spinner culture. The aggregates were then entrapped in Ca-alginate gel beads and type-I collagen gels, often used for cell immobilization [2, 5, 6, 10], to evaluate the effect of DA on liver-specific functions of hepatocytes and to determine the optimum DA of porcine hepatocytes for the cell source of BAL systems.

Materials and Methods

Chemicals

Epidermal growth factor, antibiotics, collagenase (hepatocyte-qualified), and trypsin inhibitor were purchased from Gibco BRL (Grand Islands, NY, U.S.A.), and antibodies for porcine albumin assay were from Bethyl Lab Inc. (Montgomery, TX, U.S.A). Collagen solution (Type I-A, 0.3% w/v, Cellmatrix) was from Nitta Gelatin (Osaka, Japan), and insulin, hydrocortisone, Williams' medium E, and all other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

*Corresponding author
Phone: 82-2-2260-3365; Fax: 82-2-2271-3489;
E-mail: jkpark@dongguk.edu

Isolation of Primary Porcine Hepatocytes

Primary porcine hepatocytes were harvested from slaughterhouse organs by the two-step collagenase perfusion method as described by Koebe *et al.* [7]. Livers from Landrace porcine (both genders, weighing 80–100 kg) were transported to the laboratory within 2 h in cold buffer containing 8 g/l NaCl, 0.4 g/l KCl, 0.078 g/l $NaH_2PO_4(2H_2O)$, 0.151 g/l $Na_2HPO_4(12H_2O)$, 2.38 g/l HEPES, 0.35 g/l $NaHCO_3$, 0.9 g/l glucose, 100 unit/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B. The terminal part of the left medial liver lobe (about 100 g mass) was perfused via the hepatic vein with oxygenized perfusion buffer (transport buffer plus 0.19 g/l ethylenediamine tetraacetic acid, EDTA) for 10 min at a flow rate of 100 ml/min. Then, the lobe was perfused with EDTA-free collagenase buffer containing 0.5 g/l collagenase, 0.05 g/l trypsin inhibitor, 8 g/l NaCl, 0.56 g/l $CaCl_2$, 0.4 g/l KCl, 0.078 g/l $NaH_2PO_4(2H_2O)$, 0.151 g/l $Na_2HPO_4(12H_2O)$, 2.38 g/l HEPES, 0.35 g/l $NaHCO_3$, 100 unit/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B for 10 min at the same flow rate. After cell collection, the liver cells were filtered through a cotton gauze and 150-μm nylon mesh. Nonparenchymal cells were removed by three cycles of low rpm centrifugation (500 rpm, 2 min). The average yield of viable hepatocytes was 4 to 5×10^8 per 100 g liver lobe, and viability assessed by trypan blue dye exclusion method was more than 95%.

Suspension Culture of Porcine Hepatocytes

The culture medium employed for all cultures was Williams' medium E (Gibco BRL) supplemented with the following additives: epidermal growth factor (20 μg/l), insulin (10 mg/l), $CuSO_4{\cdot}5H_2O$ (0.1 μM), $ZnSO_4{\cdot}7H_2O$ (50 pM), H_2SeO_3 (3 μg/l), linoleic acid (50 mg/l), $NaHCO_3$ (1.05 g/l), HEPES (1.19 g/l), penicillin (100 Unit/ml), streptomycin (10 mg/ml), and amphotericin B (0.25 μg/ml). This medium was named as hormonally defined medium (HDM) for the porcine hepatocyte culture.

The isolated hepatocytes were then resuspended in HDM at a cell density of 5×10^5 cells/ml. The cell suspension was placed in siliconized spinner flasks (250 ml, vertical paddle, Bellco Glass Inc., Vineland, NJ, U.S.A.) and stirred by a magnetic stirrer (Bellco Co.) at 60 rpm in a humidified 95% air/5% CO_2 incubator at 37°C. After 8 and 24 h of culturing, the medium was replaced with fresh HDM. Under these culture conditions, hepatocytes aggregated gradually and finally formed spherical aggregates within 48 h.

Entrapment of Hepatocytes

Entrapments of the hepatocytes were performed with 0, 9, 24, and 48 h of suspension culturing, using Ca-alginate bead and collagen flat gel methods. For the Ca-alginate bead method, 2% (w/v) sodium alginate solution was prepared with ultra pure water and sterilized by autoclaving. This was then mixed with 2× medium (1:1, v/v) to make a 1% (w/v) solution. Hepatocytes from the spinner flask were mixed with the alginate solution at a density of 1.5×10^6 cells/ml and were extruded through a 21G blunt-end needle with an air flow rate of 16 l/min and dropped into vigorously stirred 100 mM $CaCl_2$ solution, containing 10 mM HEPES and 20 mM D-fructose for the pH and osmolality adjustments. Under these conditions, the size of the Ca-alginate beads ranged from 1 to 1.5 mm. After stirring for 30 sec, the beads were meshed and washed with phosphate buffered saline and placed into a 6-well plate (Deep-well, 35 mm, Nunc, Denmark). The plates were then shaken on an orbital shaker (Branstead/Thermolyne, Dubuque, IA, U.S.A.) in a CO_2 incubator. Entrapped 1.5×10^6 hepatocytes were seeded in each well with 3 ml of HDM. The medium was changed daily.

For the collagen floating gel method, hepatocytes with different DA were suspended in a collagen gel solution (8 parts collagen solution: 1 part 10× medium: 1 part buffer containing 50 mM NaOH, 20 mM HEPES, and 25 mM $NaHCO_3$) at 1.5×10^6 cells/ml of cell density. This solution was then pipetted into the 12-well plate with 1 ml/well (1.5×10^5 cells/well) and incubated for 20 min to accelerate the gelation process. The flat collagen gels were transferred to a 6-well plate with 3 ml of HDM and then cultured under the same conditions as the Ca-alginate bead system. Spent medium was stored at −20°C prior to the analysis of liver specific functions.

Measurement of Hepatocytes Viability

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Co.) analysis was performed to determine the viability of the hepatocytes with various DAs as previously described [10]. The spent medium was removed and 3 ml of 0.03% (w/v) MTT solution in PBS was added to each well and incubated for 4 h. The MTT analysis medium was removed and 3 ml of isopropyl alcohol (Oriental Chemical Co., Seoul, Korea) acidified with 0.04 N HCl (Junsei Chemical Co., Tokyo, Japan) was added. This extraction was done at room temperature for 4 h. Absorbances of the extracted solution were determined at 570 nm by a UV-VIS spectrophotometer (Smart plus 2605, Young-Hwa Co., Seoul, Korea).

Measurement of Ammonia Removal Activity

To assess the ammonia metabolism of cultured hepatocytes, a medium containing 1 mM ammonium chloride was used after 2 days of culturing. The medium was changed daily and the spent medium was stored at −20°C prior to the measurement. Ammonia concentrations in the media were measured colorimetrically using the indophenol method as previously described by Kim *et al.* [5].

Measurement of Albumin Concentration

Porcine albumin contents were determined by the ELISA with purified albumin, antibody to pig albumin, and

peroxidase-conjugated antibody, and were quantitated at 490 nm with an ELISA reader (Ceres UV900HDi, Bio-Tek Instrument). The concentrations of albumin were calculated from a standard curve generated for each ELISA plate. Generally, duplicate wells were averaged for each sample.

Statistical Analysis

Data for ammonia removal rates and albumin secretion rates were statistically evaluated using Student's *t*-test. Data are given as means±SD. The difference between the means was considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Morphology of Porcine Hepatocytes Cultured Suspension

Spinner vessels were chosen to obtain porcine hepatocyte aggregates with the consideration of scale-up production for practical application of a BAL system. Suspended hepatocytes cultured in a spinner flask formed multicellular spherical aggregates within 48 h. These spheroids exhibited a tightly packed morphology and smooth surfaces. The aggregation process is shown in Fig. 1. The initial cell suspension contained a considerable amount of hepatocyte aggregates, which might have resulted from incomplete digestion of liver tissues at the isolation step (Fig. 1A) and longish aggregates of twenty to thirty cells per aggregate appeared at 9 h of culturing (Fig. 1B). Irregular rugged aggregates (size 75±28 μm) and smooth surface spheroids (size 140± 23 μm) formed within 24 h (Fig. 1C) and 48 h (Fig. 1D) of suspension culturing, respectively. This aggregation rate was

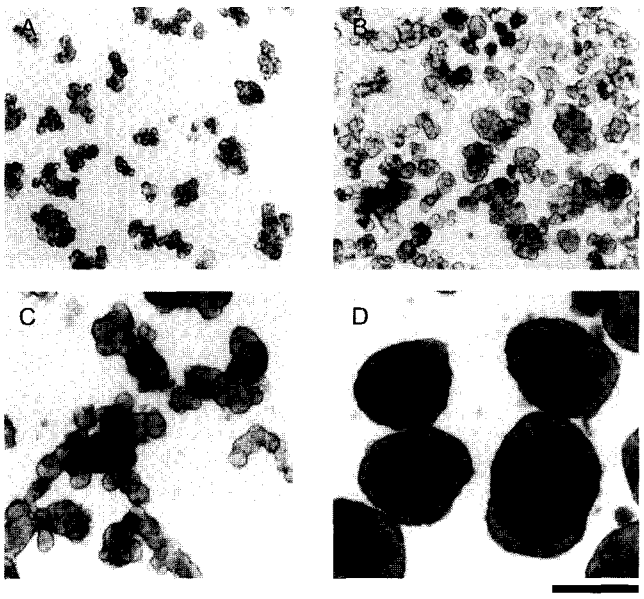

Fig. 1. Micrographs of porcine hepatocytes cultured as a suspension in a spinner flask.
A: 0 h, B: 9 h, C: 24 h, and D: 48 h. Bar indicates 100 μm.

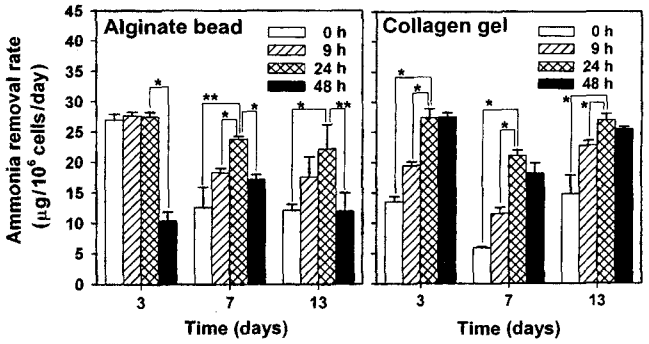

Fig. 2. Ammonia removal activities of porcine hepatocytes with various aggregation times entrapped in Ca-alginate and collagen gels.
Data are shown as the mean±standard deviation for six cultures from two independent isolations. $*P \leq 0.01$, $**P \leq 0.05$.

similar to the rat and pig hepatocyte aggregation results of Yang *et al.* [17] and Lazer *et al.* [9], respectively, and faster than the pig hepatocyte result of Darr and Hubel [1]. In the results of Darr and Hubel, the average diameter of 24 h-aggregates was only 30±16 μm. This difference might have been due to the fact that the aggregation rate of primary hepatocytes varies depending on culture circumstances such as agitation speed, media components, cell density, etc. When the degree of cell-cell contact was enriched and aggregation progressed, oxygen and nutrient diffusion became restricted. In the liver, hepatocytes exist as one-cell-thick plates along sinusoids without definite support of a collagenous matrix. Therefore, hepatocytes in spheroids have much more homogeneous cell contact than that *in vivo*, except for the cells located at the surface of the spheroids. Hence, it could be postulated that the optimum aggregation exists between dispersed single cells and round spheroids.

To investigate the effects of DA, hepatocyte aggregates were taken from the spinner culture at 0, 9, 24, and 48 h of suspension culturing. The aggregates were entrapped in either Ca-alginate beads or collagen floating gels.

Liver-Specific Functions of Hepatocytes Under Entrapped Condition

The hepatocyte aggregates were entrapped in Ca-alginate beads and collagen gel to mimic the same conditions as in the bioreactor of a BAL system. By entrapping the hepatocytes, further aggregation could also be prevented. As shown in Fig. 2, ammonia removal and albumin secretion rates from each of the hepatocyte aggregates with different DAs were determined. Since culture media containing 1 mM ammonium chloride (14 μg ammonia nitrogen/ml) were changed daily and cell density was 5×10^5 cells/ml (1.5×10^6 cells/well and 3 ml media/well), the upper limit of the ammonia removal rate was 28 μg ammonia N/10^6 cells/day. At day 3, most of the added ammonia was removed by 0, 9, 24 h aggregated hepatocytes in Ca-alginate gel beads

Fig. 3. Micrographs of porcine hepatocytes spheroid immobilized in a type-I collagen gel and cultured for 10 days.
Hepatocyte aggregates in collagen gels proceeded dendritic processes (arrow) by forming a bile duct-like structure. Bar indicates 100 μm.

and 24 and 48 h aggregated hepatocytes in the collagen gel system. The removal rate of 48 h-aggregates was less than a half of the 24 h-aggregates in Ca-alginate gel beads. In both of the encapsulation systems, hepatocytes aggregated for 24 h always showed higher ammonia removal rates. At days 7 and 13, 24 h-aggregates of hepatocytes also showed higher ammonia removal rates than any other aggregates. Ammonia removal rates of all the hepatocyte aggregates embedded in collagen gel were higher at day 13 than day 7, but not in the Ca-alginate bead system.

Unlike the hepatocyte aggregates immobilized in Ca-alginate gel beads, hepatocyte aggregates in collagen gels showed active morphological responses, such as extension of dendritic processes from the aggregates into the surrounding collagen matrix, within a few days (Fig. 3). This morphogenesis was observed in all the aggregates in collagen gels and continued throughout the culturing period. This phenomenon may be related to the recovery of ammonia removal activity of hepatocytes in collagen gel, however, the effects of the morphogenesis on liver-specific activities of the hepatocyte have hardly been studied. Only the observations of morphogenesis are reported, when hepatocyte aggregates or islets were embedded within a collagen gel and cultured in the medium containing both epidermal growth factor (EGF) and insulin [14]. The collagen and hormones enhanced many dendritic processes, which were positive for cytokeratin 19, a marker for bile duct cells [12]. Therefore, additional studies are needed to find the correlation between the hepatocyte morphogenesis and hepatic functions.

Similar trends in albumin secretion rates were also observed with the hepatocyte aggregates immobilized in Ca-alginate gel beads and the collagen gel entrapment system (Fig. 4). Albumin secretion rates of hepatocytes aggregated for 24 and 48 h were higher than those aggregated for 0 and 9 h in both types of immobilization systems at days 3 and 7. The plasma protein secretion rates of the immobilized hepatocytes were increased as culturing progressed, except for 48 h-aggregates. At day 13 of culturing, the round large spheroids formed at 48 h-aggregation showed a significant decrease in albumin secretion rates from that of day 7 in Ca-alginate bead (P=0.013) and collagen gel (P=0.022) systems. Therefore, it was 24 h-aggregated hepatocytes that constantly secreted higher amount of albumin into the media.

Hepatocytes Viability

To assess the influence of cell-cell contact on the viability of the cultured hepatocytes, a MTT assay was performed after 13 days of culturing in both immobilization systems, and the viability was expressed as a relative viability to 24 h hepatocyte aggregates in collagen gel. Each data point represents the average of six samples from two independent isolations. As shown in Fig. 5, the hepatocyte aggregates cultured in collagen gel showed significantly different viabilities with DA of hepatocytes. The differences were greater than those of functional activities, such as ammonia removal and albumin secretion. This indicated that tissue-specific functions and cell viability measured by MTT conversions did not have a proportionate correlation. However, hepatocytes aggregated for 24 h showed maximum viability and functional activities in both types of immobilization systems.

The clinical and commercial application of the BAL technology relies on the establishment of a safety-qualified and quality-controlled large cell-bank of hepatocytes. Therefore,

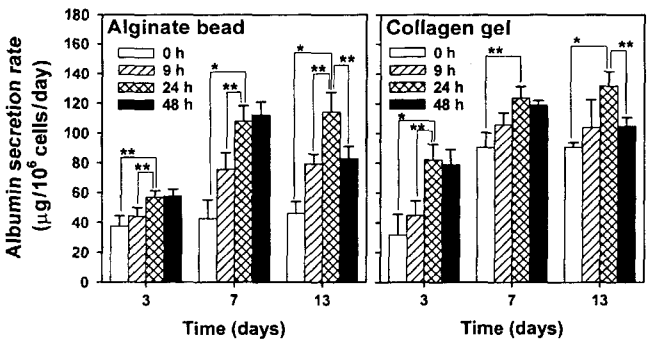

Fig. 4. Albumin secretion rates of porcine hepatocytes with various aggregation times entrapped in Ca-alginate and collagen gel.
Data are shown as the mean ± the standard deviation for six cultures from two independent isolations. *P≤0.01, **P≤0.05.

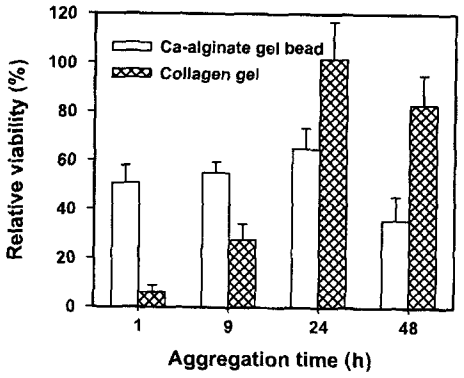

Fig. 5. Relative viability of porcine hepatocytes with various aggregation times entrapped in Ca-alginate and collagen gel.
MTT viability assays was performed at 13 days of culturing. Data are shown as the mean ± the standard deviation for six cultures from two independent isolations.

the efficient cryopreservation technique of hepatocytes is essential. It was recently reported that hepatocytes suspended for 24 h prior to being frozen showed a higher post-thaw albumin secretion rate and viability than either frozen immediately after isolation or after 48 h suspension cultures [1]. Therefore, it was proven again in the present study that the hepatocytes aggregated for 24 h are the optimum aggregates for the development of a BAL system.

In this study, spinner vessels were used for the aggregation of hepatocytes. Therefore, the aggregation process was easily scaled-up by using a large-scale commercial mammalian cell bioreactor with oxygenizing equipment. Thus, hepatocytes aggregated for 24 h by suspension culture could offer a stable cell source of a BAL system for hepatic functions and convenience scale-up.

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