A method for the effective formation of hepatocyte spheroids using a biodegradable polymer nanosphere

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Abstract: Cultures of hepatocytes in spheroid form are known to maintain higher cell viability and exhibit better hepatocyte functions than those in monolayer cultures. In this study, a method for the formation of hepatocyte spheroids was developed using biodegradable polymer nanospheres. The addition of poly(lactic-*co*-glycolic acid) nanospheres to hepatocyte cultures in spinner flasks increased the efficiency of hepatocyte spheroid formation (the number of cells in spheroids divided by the total cell number) as compared with hepatocyte cultures without nanospheres (control). The viability and mitochondrial activity of the hepatocyte spheroids in the nanosphere-added cultures were significantly higher than those in the control. In addition, the mRNA expression levels of albumin and phenylalanine hydroxylase, both of which are hepatocyte-specific proteins, were significantly higher in the nanosphere-added cultures than in the control. This new culture method improves upon the conventional method of forming hepatocyte spheroids in terms of spheroid formation efficiency, cell viability, and hepatocyte function. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 268–275, 2006

Key words: hepatocyte; poly(lactide-*co*-glycolide) nanosphere; spheroid

INTRODUCTION

Liver transplantation has significantly improved the treatments for end-stage liver disease and metabolic liver disease. However, liver transplantation has a number of limitations, including a serious donor shortage, fatal surgical complications, and the necessity of life-long immunosuppression. Hepatocyte transplantation and bioartificial liver systems could be an alternative to the more traditional therapeutic approach and overcome the limitations of liver transplantation.[1–4] Hepatocyte transplantation is a simpler and less costly method than liver transplantation. The shortage of donors could also be solved, since the transplantation of hepatocytes permits the use of a single donor organ for multiple recipients, and cryopreservation of the hepatocytes makes future use possible.[5] The transplantation of hepatocytes differentiating from autologous bone marrow stem cells could eliminate the requirement of life-long immunosuppression.[6] Bioartificial liver support devices have been developed, which replace the synthetic, metabolic, and detoxification functions of the liver. Some such devices have already been evaluated in clinical trials.[2,7,8] Importantly, hepatocytes used in hepatocyte transplantation and bioartificial liver systems must maintain high cell viability and hepatocyte-specific functions.

The ability to culture hepatocytes in a spheroid form is advantageous over the conventional monolayer cultures because it maintains high cell viability and hepatocyte-specific functions.[9,10] Hepatocyte spheroids sustain a high cell viability over the culture period.[10] Hepatocytes in spheroids are morphologically and ultrastructurally similar to hepatocytes in *in vivo* liver tissue and enhance albumin synthesis, urea genesis, and cytochrome P450 activity, as compared with monolayer cultures.[9–12] The maintenance of hepato-

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cyte functions in hepatocyte spheroids is attributed to close cell–cell contact, the reestablishment of cell polarity, and the formation of bile canaliculi-like channels.[13] In contrast, hepatocytes in monolayer cultures rapidly lose their hepatocyte functions, such as albumin synthesis, ureagenesis, and cell viability.[14] Because of the advantages of the hepatocyte spheroids, they have been studied for hepatocyte transplantation[15] and bioartificial liver systems.[16]

Conventionally, the formation of hepatocyte spheroids has been induced by a suspension culture, using a petri dish or spinner flask.[10,17–20] In this study, we developed a novel method for the formation of hepatocyte spheroids, using nanospheres fabricated from a biodegradable, biocompatible polymer. This polymer causes no or mild inflammatory reactions when implanted for cell transplantation.[21] We investigated whether the nanosphere method promoted the efficiency of hepatocyte spheroid formation and improved the cell viability and hepatocyte-specific gene expression as compared with the conventional method of hepatocyte spheroid formation.

MATERIALS AND METHODS

Nanosphere fabrication

Biodegradable poly(lactic-*co*-glycolic acid) (PLGA) nanospheres were prepared using the oil/water emulsion and solvent evaporation–extraction method, as previously described.[22–24] In brief, 400 mg of 75:25 PLGA (molecular weight = 100,000 Da, Birmingham Polymers, Birmingham, AL) was dissolved in 4 mL of methylene chloride, added to 100 mL of aqueous 2% (w/v) polyvinyl alcohol (Sigma, St. Louis, MO), and emulsified for 90 s using a sonicator (60 W, Sonic Dismembrator 550, Fisher Scientific, Fair Lawn, NJ). After evaporation at 4°C overnight, the nanospheres were collected by centrifugation at 5000 rpm for 20 min, washed three times with distilled water, and lyophilized for 2 days. PLGA nanospheres containing a fluorescent dye (6-coumarin, Polysciences, Warrington, PA) were also prepared by adding the dye to the PLGA solution to examine the distribution of PLGA nanospheres in the hepatocyte spheroids. The size distribution of the fabricated PLGA nanospheres was determined by examining scanning electron microscopy (SEM; JSM-6330F; JEOL, Tokyo, Japan) images of the nanospheres.

Hepatocyte isolation

Hepatocytes were isolated from female, 5-week-old ICR mice (Jung-Ang Lab Animal, Seoul, Korea), using a modification of the previously described two-step collagenase perfusion technique.[25] Mice were anesthetized and a mid-line laparotomy was performed. After a 24-gauge catheter needle was inserted into the portal vein, the liver was perfused with a preperfusion solution containing ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (2.5 m*M*, Sigma) and then perfused with a collagenase solution (0.05 mg collagenase/mL in perfusion solution, Gibco BRL, Grand Island, NY) at 37°C. The digested liver was excised, and then the cells were released from the digested liver by gentle shaking in Williams medium E (Gibco BRL) supplemented with 1% (w/v) penicillin–streptomycin (Gibco BRL). The isolated cells were filtered through a 40 μm nylon mesh and collected after centrifugation at 500 rpm for 3 min.

Spheroid culture

Spinner flasks (Bellco, Vineland, NJ) were siliconized before use to prevent cell adhesion to the flask walls. Isolated hepatocytes were inoculated into 100-mL spinner flasks containing 30 mL of culture medium at a cell concentration of 10^5 cells/mL and stirred at 50 rpm with or without nanospheres (0.3 mg/mL). The culture medium used was Williams medium E supplemented with 1% (w/v) penicillin–streptomycin, epidermal growth factor (20 μg/L, Sigma), and insulin (1 μg/L, Sigma). The medium was changed every 2 days. All cultures were grown in triplicate.

Quantitative analyses of the hepatocyte spheroids

The size of the hepatocyte spheroids was determined by analyzing at least 100 spheroids from a minimum of 10 individual photomicroscope images. The efficiency of hepatocyte spheroid formation was calculated by dividing the number of cells in the spheroids by the total cell number. Hepatocyte spheroids were collected after settling. The number of hepatocytes in spheroids was measured after the dissociation of the hepatocyte spheroids into single cells with 0.1% (w/v) trypsin (Sigma).

Scanning electron microscopy

For examination by SEM, the hepatocyte spheroids were fixed in 1% (v/v) glutaraldehyde and 0.1% (v/v) formaldehyde for 1 and 24 h, respectively, dehydrated with a series of graded ethanols, and dried. The dried samples were mounted on aluminum stubs, using double-sided carbon tape, then sputter-coated with gold, and examined by SEM.

Histological analyses

For histological analyses, the spheroids were fixed in a 10% (v/v) buffered formaldehyde solution, dehydrated with a series of graded alcohol solutions, and embedded in paraffin. The specimens were cut into 4-μm thick sections and processed with hematoxylin and eosin (H&E) staining. For

immunohistochemical analyses, deparaffinized 4-μm thick sections were stained using antibodies against hepatocyte (Clone OCH 1E5, DAKO, Carpenteria, CA), and treated with a secondary antibody, rhodamine-conjugated anti-mouse IgG (Jackson Immunoresearch. West Grove, PA). The immunofluorescently stained sections were examined by confocal microscopy (LSM510, Carl Zeiss, Oberkochen, Germany).

Viability assay

The viability of cultured cells was evaluated by the Neutral Red assay.[26,27] The neutral red used in this study is a chemosensitive assay of cell viability. The neutral red is a weak cationic dye that readily penetrates viable cell membranes by nonionic diffusion, and it is accumulated intracellularly in lysosomes. The process requires intact membranes and active metabolism in the cell. Failure to take up neutral red indicates that the cell has suffered damage. The dye taken up by the cells is subsequently extracted and measured. It is thus possible to distinguish between viable or dead cells. A suspension of single cells and spheroids in 5 mL of medium per spinner flask was rinsed with phosphate-buffered saline (PBS, Sigma), and 500 μL of the Neutral Red solution [0.005% (w/v) in culture medium, Sigma] was added. After incubation for 3 h at 37°C, the Neutral Red solution was removed and a dye extraction was performed by adding 500 μL of 1% (v/v) acetic acid in 50% (v/v) ethanol solution. The absorbance was measured at 540 nm using an ELISA plate reader (Powerwave X340, Bio-TEK Instruments, Winooski, VT).

The mitochondrial metabolic activity of the cells was determined using the MTT assay. Briefly, a suspension of single cells and hepatocyte spheroids was rinsed three times with PBS, and 100 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 2 mg/mL in PBS, Sigma) was added. After incubation for 4 h at 37°C, the MTT solution was removed. The resulting insoluble particles were dissolved in 100 μL of dimethyl sulfoxide hybrid-max (Sigma), and the absorbance was measured at 540 nm using an ELISA plate reader. The percentages of cell viability and metabolic activity were calculated by normalizing the values with the absorbances of the freshly isolated hepatocytes.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cell spheroids, using the TRIzol reagent (Invitrogen, Carlsbad, CA). Cell spheroids were lysed with 1 mL of the TRIzol reagent, added to 200 μL of chloroform, and centrifuged at 12,000 rpm for 15 min. The RNA pellet was washed with 70% (v/v) ethanol, dried, and dissolved in diethyl pyrocarbonate-treated water. The reverse transcription reaction was performed with 5 μg of pure RNA, using SuperScript™ II reverse transcriptase (Invitrogen). Synthesized cDNA was amplified by PCR, using the following primers: (1) albumin; sense 5′-TGC TGC TGA TTT TGT TGA GG-3′, antisense 5′-GCT CAC TCA CTG GGG TCT TC-3′. (2) phenylalanine hydroxylase (PAH); sense 5′-ACT TGT ACT GGT TTC CGC CT-3′, antisense 5′-AGG TGT GTA CAT GGG CTT AG-3′. (3) β-actin; sense 5′-CCC ACA CTG TGC CCA TCT AC-3′, antisense 5′-AGT ACT TGC GCT CAG GAG GA-3′. The PCR products were visualized by electrophoresis on 2% (w/v) agarose gels containing ethidium bromide. The visualized bands were photographed, and the images were scanned and saved as tagged image files, using Adobe Photoshop Software (Adobe Systems, Mountain View, CA). The densitometric analysis of the scanned images was carried out using the Scion Image program (NIH image, Scion Corporaton, MD). β-actin was used as the "housekeeping" mRNA control. The RT-PCR assay was performed using three samples per group. The ratios of albumin/β-actin and PAH/β-actin were statistically analyzed.

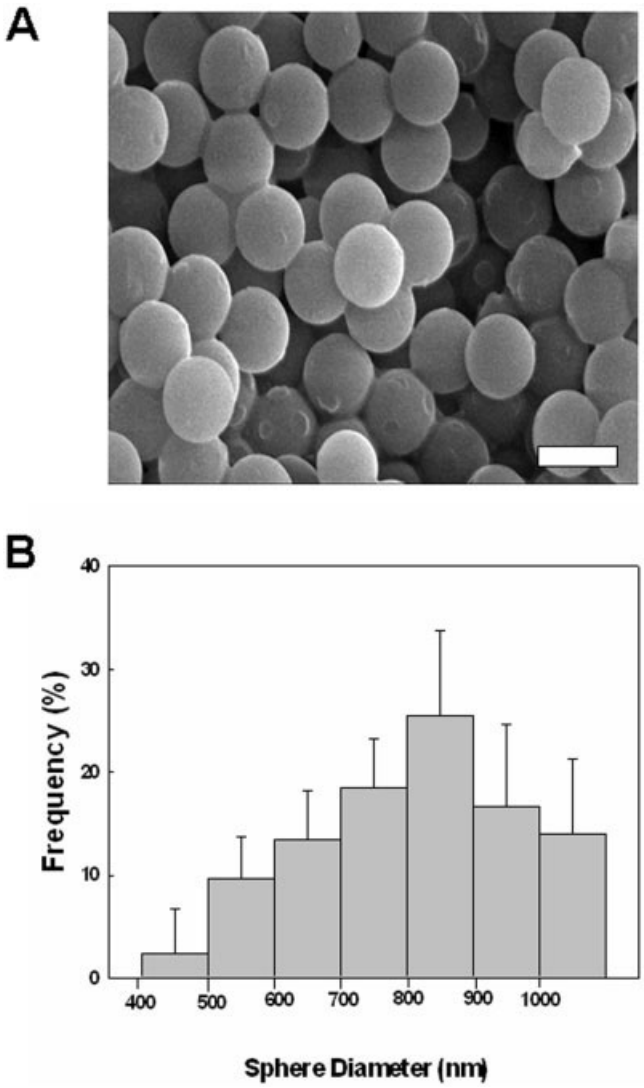

Figure 1. A: SEM image of fabricated PLGA nanospheres and B: distribution of the nanosphere diameter. The scale bar indicates 1 μm.

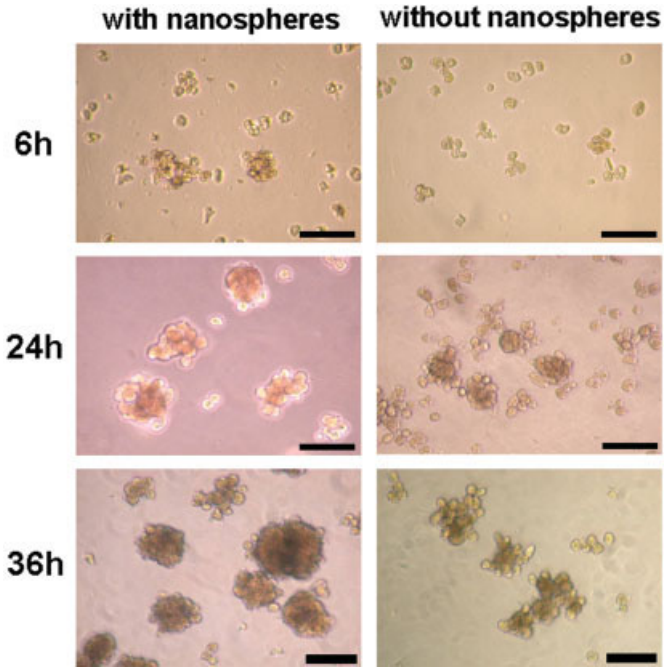

Figure 2. Light microscopic photomicrographs of hepatocyte spheroids in suspension cultures with and without nanospheres at 6, 24, and 36 h. The scale bars indicate 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Statistical analysis

Quantitative data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the unpaired Student's t test on InStat software (InStat 3.0, GraphPad Software, San Diego, CA). A value of $p<0.05$ was considered to be statistically significant.

RESULTS

The fabricated PLGA nanospheres were spherically shaped and had a smooth surface [Fig. 1(A)]. The diameter of the nanospheres was 835 ± 173 nm [Fig. 1(B)]. The addition of nanospheres to a stirred cell suspension induced accelerated hepatocyte spheroid formation. Hepatocytes cultured with nanospheres started to form spheroids at 6 h, whereas hepatocytes cultured without nanospheres (control cultures) did not form spheroids at 6 h (Fig. 2). The size of the hepatocyte spheroids was larger in the nanosphere-added cultures than in the control cultures at all time points. After 24 h of incubation, the spheroid diameter was 69.0 ± 7.6 μm in the nanosphere-added cultures, whereas only a few spheroids that had a diameter of 39.4 ± 8.5 μm existed in the control cultures (Fig. 3). After 36 h of incubation, the hepatocyte spheroid diameter was 87.7 ± 10.3 μm and 56.6 ± 7.4 μm in the nanosphere-added cultures and the control cultures, respectively.

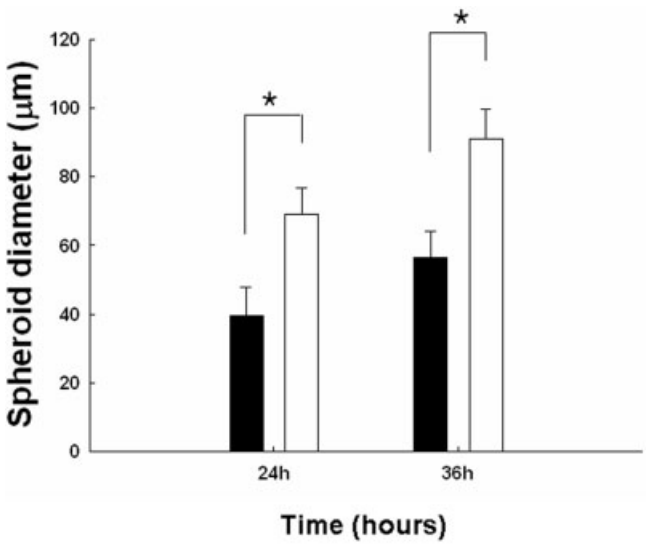

Figure 3. The diameter of hepatocyte spheroids in cultures with nanospheres (blank bars) and without nanospheres (solid bars) at 24 and 36 h. The data represent the mean ± SD ($n = 7$). *$p<0.05$.

A higher proportion of the cells formed spheroids in the nanosphere-added cultures than the control cultures. The portion of cells incorporated into the spheroids (efficiency of spheroid formation) in the nanosphere-added culture group was (24.6 ± 1.5)% at 6 h and increased to (81.1 ± 3.9)% at 36 h, whereas the portion in the control spheroids was only (43.9 ± 5.5)% at 36 h (Fig. 4). A histological analysis of the spheroids showed no significant difference in the histological structure of the spheroids between the two

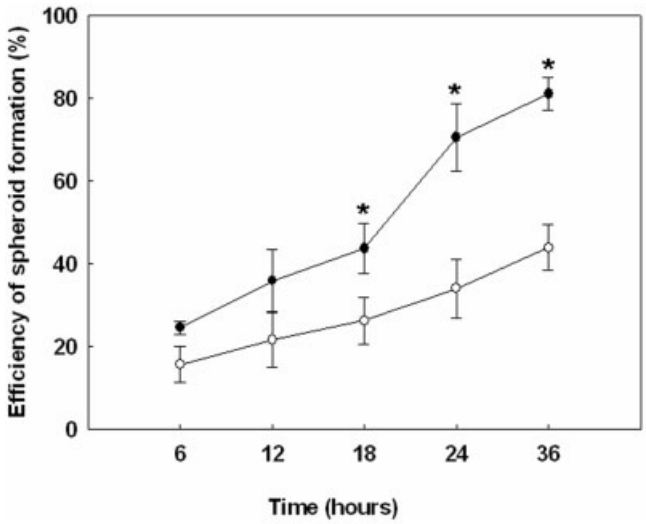

Figure 4. The efficiency of spheroid formation in suspension cultures with nanospheres (solid circles) and without nanospheres (blank circles). The data represent the mean ± SD ($n = 7$). *$p<0.05$.

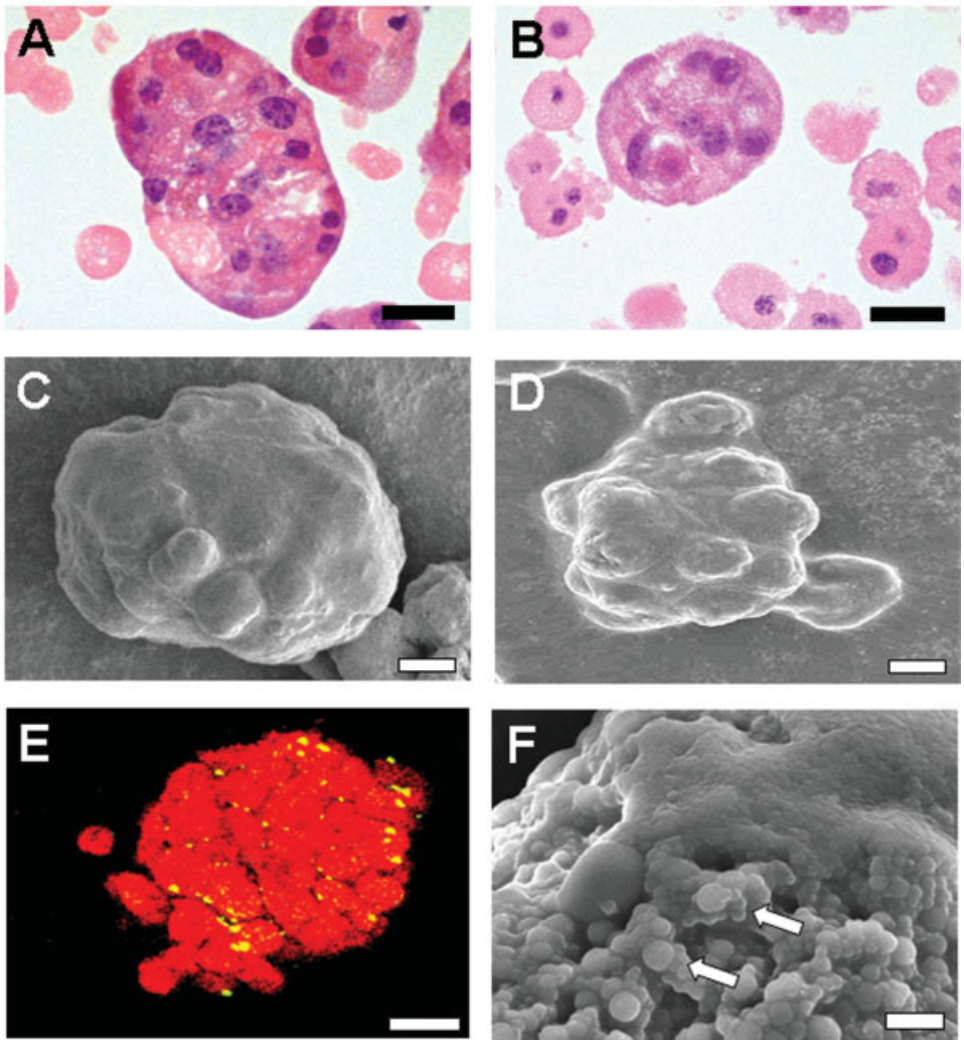

Figure 5. Hepatocyte spheroids in spinner cultures at 36 h. Histological sections (H&E staining) of the spheroids in the suspension cultures. A: with nanospheres and B: without nanospheres. SEM photographs of hepatocyte spheroids. C, F: with nanospheres and D: without nanospheres. E: A confocal micrograph of hepatocyte spheroids in culture with 6-coumarin-loaded nanospheres. The red color indicates positive staining with antibodies against hepatocyte. The yellow color indicates PLGA nanospheres. F: A higher magnification SEM photograph of the spheroid in culture with nanospheres. The arrows indicate the nanospheres. The scale bars indicate 20 μm (A–E) and 5 μm (F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

groups [Fig. 5(A,B)]. An examination by SEM showed the adhesion of multiple cells in spherical shapes [Fig. 5(C,D)]. PLGA nanospheres were evenly distributed throughout the hepatocyte spheroids [Fig. 5(E)], and were also present in the spheroid surface [Fig. 5(F)].

The nanosphere-added culture possessed superior viability compared with the control culture. The cell viability, as measured by the Neutral Red assay, was significantly higher ($p<0.05$) in the nanosphere-added cultures than in the control cultures [Fig. 6(A)]. Mitochondrial activity was also significantly higher ($p<0.05$) in the nanosphere-added cultures than in the control cultures [Fig. 6(B)]. The hepatocytes in the nanosphere-added cultures showed superior hepatocyte-specific gene expression compared with the cells in the control cultures. Semiquantitative RT-PCR analysis showed that the mRNA expression levels of albumin and PAH, both of which are specifically expressed in hepatocytes, were significantly higher ($p<0.05$) in the nanosphere-added cultures than in the control cultures at 36 and 72 h (Fig. 7).

DISCUSSION

Conventional hepatocyte culture techniques include a monolayer culture using a cell culture dish and a suspension culture using a petri dish or spinner flask.[10,17–20,28] In hepatocyte cultures, the formation of hepatocyte spheroids may be critical for the survival and differentiated functions of the hepatocytes be-

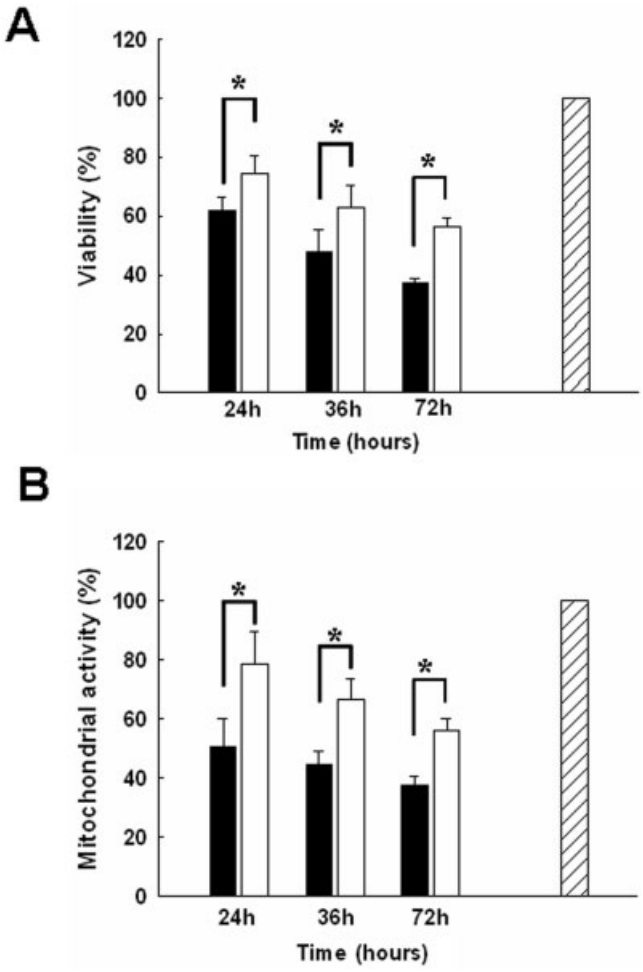

Figure 6. A: The viability and B: mitochondrial activity of hepatocyte spheroids cultured with nanospheres (blank bars) and without nanospheres (solid bars), normalized with the viability, and mitochondrial activity of freshly isolated hepatocytes (lined bars), respectively. The viability and mitochondrial activity were determined with the Neutral red and MTT assays, respectively. The data represent the mean ± SD ($n = 7$).

cause hepatocytes in monolayer or in single cell suspension have been found to rapidly die or lose their hepatocyte-specific functions.[22–24] In this study, we investigated the effect of nanospheres on the formation of hepatocyte spheroids in suspension cultures of hepatocyte cells. The results showed that the nanosphere-added cultures showed enhanced hepatocyte spheroid formation, hepatocyte viability, and hepatocyte-specific gene expression as compared with the culture without nanospheres.

The use of nanospheres promoted the rate and efficiency of spheroid formation and cell viability compared with the control cultures. Hepatocyte spheroids ranging from 50 to 100 μm were formed between 2 and 3 days in the conventional method.[18,20] In the present study, hepatocyte spheroids in the nanosphere method formed between 24 and 36 h. The higher cell viability in the nanosphere-added cultures may be due to the increased speed at which the spheroids formed and the higher efficiency of hepatocyte spheroid formation, since single hepatocytes in suspension die rapidly.[18,24] Our previous studies have demonstrated that the formation of cell spheroids using nanospheres enhanced the rate of cell aggregation, subsequent growth of the aggregated cells, and the production of a recombinant protein more than the cell spheroid formations without nanospheres.[22–24] It has been shown that hepatocyte spheroids have a tissue-like morphology that includes cell–cell interaction, a tight junction, and networks of microvilli-lined channels.[29] The microvilli-lined channels help to maintain the viability of the cells in the interior by facilitating the diffusion of oxygen and nutrients.

The nanosphere-added cultures improved the hepatocyte-specific gene expression of the cultured hepatocytes compared with the control cultures. The nanosphere-added culture maintained the mRNA expression levels of albumin and PAH compared to control culture for long time (72 h). The mRNA expression of albumin and PAH, both of which are hepatocyte-specific proteins,[30] in the nanosphere-added cultures was significantly enhanced compared with that in the control cultures. It has been shown that hepatocyte cultures in spheroid form enhanced hepatocyte-specific gene expression compared with the monolayer cultures.[28] The enhanced gene expression in the spheroid cultures may be due to the establishment of tight cell–cell contact and membrane reorganization, including polarization.[29] The enhanced hepatocyte-specific gene expression in the nanosphere-added cultures may be caused by the enhanced formation of spheroids in the nanosphere-added cultures as compared with the control cultures. The higher cell viability in the nanosphere-added cultures may also contribute to the enhanced hepatocyte-specific gene expression.

A potential problem in cell spheroid cultures is necrosis in the core of the cell spheroids. Spheroids with a diameter larger than 200 μm would have diffusional limitations in the transport of nutrients and metabolites, which would result in necrosis in the spheroid core.[31] In the nanosphere-added cultures, an average spheroid diameter of less than 88 μm was maintained for the entire culture period, which is adequate to maintain viable cells.

In conclusion, the use of biodegradable polymer nanospheres in suspension cultures of hepatocytes promoted hepatocyte spheroid formation, hepatocyte viability, and hepatocyte-specific gene expression. This method might also be useful for the culturing of other types of cells that require spheroid formation. The use of PLGA nanospheres as a cell culture and transplantation matrix could be extended to deliver

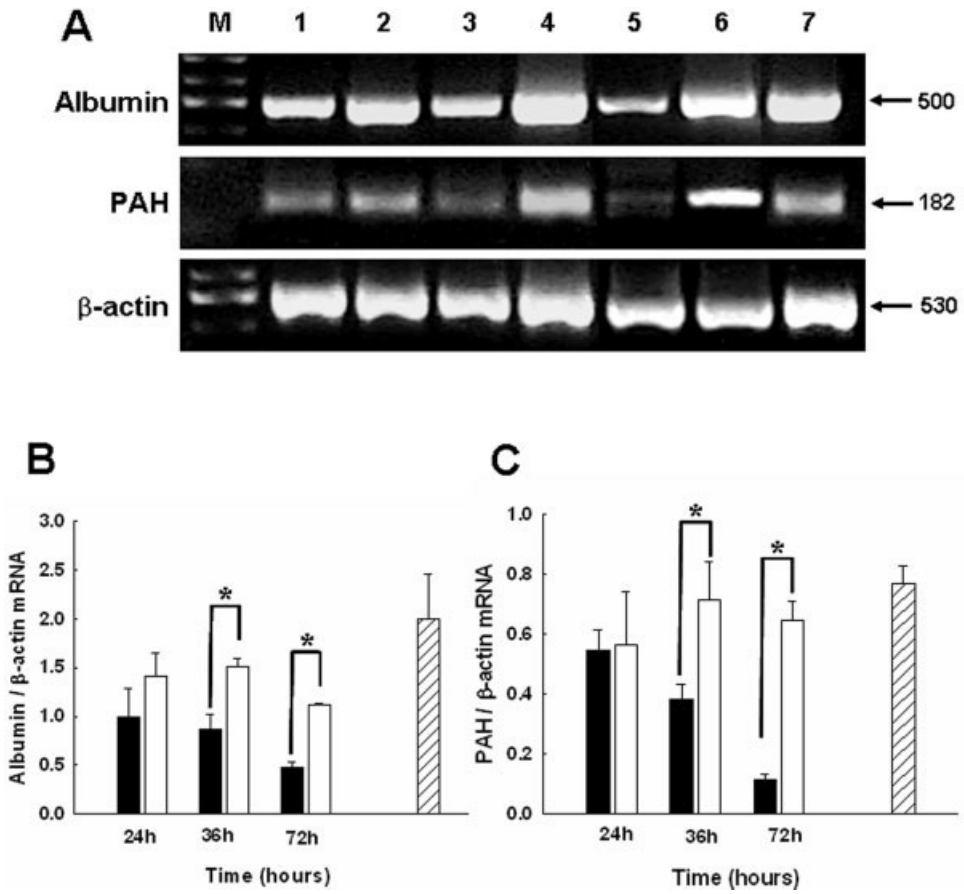

Figure 7. A: Semiquantitative RT-PCR analyses of albumin and PAH. (M, 1kb DNA molecular weight marker; lane 1, hepatocyte spheroids cultured without nanospheres at 24 h; lane 2, hepatocyte spheroids cultured with nanospheres at 24 h; lane 3, hepatocyte spheroids cultured without nanospheres at 36 h; lane 4, hepatocyte spheroids with nanospheres at 36 h; lane 5, hepatocyte spheroids cultured without nanospheres at 72 h; lane 6, hepatocyte spheroids cultured with nanospheres at 72 h; and lane 7, freshly isolated hepatocytes). The relative mRNA expression levels of (B) albumin and (C) PAH of hepatocyte spheroids with nanospheres (blank bars) and without nanospheres (solid bars), and freshly isolated hepatocytes (lined bars) normalized by mRNA expression level of β-actin ($n = 3$). *$p<0.05$.

genes and growth factors to stimulate cell growth, survival, and differentiation.[32]

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