



Original Article

Establishment of large canine hepatocyte spheroids by mixing vascular endothelial cells and canine adipose-derived mesenchymal stem cells



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ABSTRACT

Introduction: Differentiation of hepatocytes and culture methods have been investigated in dogs as a tool to establish liver transplant and drug metabolism examination systems. However, mass culture techniques for canine hepatocytes (cHep) have not been investigated, and it is necessary to construct a suitable culture system. Recently, a protocol called Bud production has attracted attention, and a mixed culture of human and mouse hepatocytes, stem cells, and artificial blood vessels significantly improved the size and formation ratio of spheroids. The purpose of this study was to investigate and improve the in vitro culture of cHep by mixing canine adipose-derived mesenchymal stem cells (cASCs) and human umbilical vein endothelial cells (HUVECs).

Methods: Spheroid formation ratio and histological examination were evaluated among four culture methods, including cHep alone, two-mix (cHep + cASCs and cHep + HUVEC), and three-mix (cHep + HUVEC + cASCs), on days 0, 4, and 7. Expression levels of liver-related genes (*ALB*, *AFP*, *α1-AT*, *CDH1*, *CYP2E1*, *CYP3A12*, and *TAT*) were evaluated by quantitative real-time polymerase chain reaction (RT-PCR). Protein expression of albumin, vimentin, and von Willebrand Factor (vWF) was investigated to confirm the location of the hepatocytes.

Results: The ratio of spheroid formation was 60.2% in the three-mix culture and was significantly improved compared with cHep alone (5.9%) and two-mix; cHep + cASCs (36.2%) and cHep + HUVEC (26.4%) ($P < 0.001$). Histological evaluation revealed that the three-mix spheroids formed large canine hepatocyte spheroids (LcHS), and hepatocytes were distributed in the center of the spheroids. Quantitative gene expression analysis of LcHS showed that liver-related genes expression were maintained the same levels with that of a culture of cHep alone from days 4–7.

Abbreviations: LcHS, large canine hepatocyte spheroids; 3D, three-dimensions; DILI, drug induced liver injury; cHep, canine primary-cultured hepatocytes; cASC, canine adipose-derived mesenchymal stem cells; HUVEC, human umbilical vein endothelial cells; HTM, hepatocyte thawing medium; HGM, hepatocyte growth medium; rf-HGF, recombinant feline hepatocyte growth factor; FBS, fetal bovine serum; SF-HGM, hepatocyte growth medium for spheroid formation; hFGF, human fibroblast growth factor; EGM, endothelial cell growth medium; MSCGM, mesenchymal stem cell growth medium; PBS, phosphate-buffered saline; ALB, albumin; α 1-AT, α 1-antitrypsin; TAT, tyrosine aminotransferase; AFP, α -fetoprotein; CDH1, cadherin-1 (epithelial-cadherin); CYP, cytochrome; iPSC, induced pluripotent stem cells; CD, cluster of differentiation.

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Conclusion: These results revealed that the three-mix culture method using cHep, HUVECs, and cASCs was capable of promoting LcHS without impairing liver function in cHep, suggesting that LcHS could be used for the application of high-volume culture techniques in dogs.

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1. Introduction

Recent studies indicate that some dogs develop a variety of spontaneous liver diseases, including hepatitis, cirrhosis, lipidosis, portosystemic shunts, and portal hypoplasia [1]. In dogs, liver fibrosis and cirrhosis concurrent with chronic hepatitis often have poor prognosis, and the pathological process is similar to that of human liver disease [2]. Some therapeutic drugs included inflammatory, antioxidant, and antifibrotic agents, and gene therapy have been used for the treatment of hepatitis and secondary liver fibrotic disease, but effective treatment has not been yet established [1,3,4]. In particular, canine hepatitis has a poor prognosis, with a median mean survival of approximately one and a half years [2]. Thus, new effective treatment methods for canine cirrhosis should be established as soon as possible, as the prognosis was reported to be only 22 days. On the other hand, in drug discovery research, the establishment of an artificial liver tissue with drug metabolizing function should be used to predict drug-induced liver injury (DILI) and metabolite production in vitro. In non-clinical studies of drug discovery studies, dogs that are biologically closer to humans than mice and rats have been subjected to safety studies. However, from the viewpoint of animal welfare, urgent development of alternative methods is required [5–8].

In recent years, studies on rats have used regenerative medicine to treat liver diseases. Li et al. reported that transplantation of differentiated-induced hepatocytes significantly improved liver tissue damage [9]. As an alternative to conventional living-donor liver transplantation, liver transplantation using primary cultured hepatocytes is also a promising therapeutic candidate [10]. However, canine allogeneic liver transplantation, including living-donor liver transplantation, has not been established in the field of veterinary medicine. In addition, a novel construct culture protocol must be established with a three-dimensional (3D) structure of canine artificial liver, given that hepatic function and metabolism such as albumin and cytochrome (CYP) expression are exerted in the liver tissue of a three-dimensional structure [11]. To solve this problem, research on constructing tissue-like structures using 3D cell culture is being actively conducted.

A plurality of methods using 3D bioprinting technology have been established for producing a 3D tissue-like structure by laminating, and many methods use scaffolding or bio-inks for lamination in humans and rats [12–15]. On the other hand, a novel culture method using sword-shaped needle was reported and concerned that a number of problems, which includes immune rejection, large amount of cost, and cell toxicity, by using scaffolding material would be solved. In fact, culture methods using sword-shaped needles have been applied in many tissues for organizational construction and have been proven to be useful in nerve cells [16–21]. However, large hepatocyte spheroids with a diameter greater than 500 μm must be established in order to apply 3D bioprinting technology using a sword-shaped needle for liver construct. Like other organs, hepatic spheroids were cultured by mixing human primary cultured hepatocytes, human umbilical vein endothelial cells (HUVECs), and adipose-derived mesenchymal stem cells (ASCs), and then laminated using a 3D bioprinter [22–24].

Recently, Takebe et al. demonstrated that the three-mix culture, composed of mesenchymal stem cells (MSCs), vascular endothelial cells, and specific hepatocyte intrahepatic embryo-like cells (iPSC-HE) derived from induced pluripotent stem cells, was capable of forming 3D hepatocyte spheroids with blood vessels (vascularized liver buds) by self-organization due to reproduction of cell–cell interactions at the time of organ development [25]. Surprisingly, in humans, 3D hepatocyte spheroids were constructed by culturing with human primary cultured hepatocytes, vascular endothelial cells, ASCs, and liver laminating tissues using these spheroids, and a 3D bioprinter was used to maintain a drug-metabolizing ability for about three months [22–24].

However, it is unclear whether these methods can be applied to dogs, given that the biological characteristics, physiological functions, and metabolic capacity of hepatocytes differ between dogs and other species. Furthermore, not only the three-mix culture, but also large spheroids using primary hepatocytes have not been reported in dogs. Thus, the purpose of this study was to evaluate spheroid formation ability and hepatic function of large cHep spheroids (LcHS) co-cultured with primary canine hepatocytes, ASCs, and HUVECs.

2. Materials & methods

2.1. Preparation of cells

Canine primary-cultured hepatocytes (cHep) were obtained from Biopredic International (Brittany, France). HUVECs were purchased from ScienCell Research Laboratories (CA, USA). Canine ASCs (cASCs) (Celltrust Animal Therapeutics Co., Ltd., Kanagawa, Japan) were used. A healthy male beagle dog was the donor of adipose tissue, which was aseptically collected from the abdominal region by surgical excision. Canine ASCs were isolated as follows; adipose tissue was digested with collagenase (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) at 50 U/0.1 g fat/mL over 1.5 h at 37 °C. After digestion, the sample was centrifuged at 750 g for 10 min. The precipitate was collected and cultured in mesenchymal stem cell growth medium (MSCGM; Lonza, Basel, Switzerland) in a humidified incubator at 37 °C in the presence of 5% CO₂. After 72 h, the cultures were washed with phosphate buffered saline (PBS; FUJIFILM Wako Pure Chemical Co., Osaka, Japan) to remove non-adherent cells, and fresh medium was added. After 7 days of culture, the cells were harvested and subcultured. Canine ASCs were cultured and expanded; cells in the first passage were used for this study. The viability of cASCs was confirmed to be greater than 90%, and cASCs were analyzed for cell surface markers, including CD 90, CD 44, CD 45, and MHC class II, by flow cytometry (CyFlow space, Sysmex Co. Ltd. Kobe, Japan). The majority of the cells were positive for CD90 and CD44, whereas CD45 and MHC class II were undetectable. Moreover, the multipotency of cASCs was estimated by differentiation into osteoblasts, adipose cells, and chondrocytes. More than 80% of the osteoblasts were positive for alkaline phosphatase staining. Cells that differentiated into chondrocytes were stained dark blue by Alcian blue staining (Alcian Blue Solution, pH2.5, FUJIFILM Wako Pure Chemical Corporation). Adipocyte-

differentiated cASCs were stained with Oil Red O (Oil Red O Staining Solution, MUTO PURE CHEMICALS CO., LTD. Tokyo, Japan) in their cytoplasm.

2.2. Culture of spheroids

The first passage cells of cHep and P2 cASCs were used to establish canine hepatocyte spheroids. These cells were thawed from the cryopreserved cells in liquid nitrogen. Human umbilical vein endothelial cells and cASCs were placed in a thermostatic bath at 37 °C for freeze thawing. These cell suspensions were decanted into pH 7.4 PBS (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 330×g for 5 min. After that, HUVECs and cASCs were washed twice with PBS and cultured with endothelial cell growth medium (EGM) and MSCGM, respectively, in a Collagen Type I-Coated Flask (25 cm²) (ACG Techno Glass, Shizuoka, Japan). Human umbilical vein endothelial cells and cASCs were precultured in EGM (Lonza) and MSCGM (Lonza), respectively. The medium was replaced every 48 h during the culture period. For HUVECs and cASCs, the medium was discarded on the fourth day of culture, the culture surface was washed twice with PBS, and Accutase (Innovation Cell Technologies, CA, USA) was added to the culture vessel and incubated at 37 °C for 10 min. The suspension containing the stripped cells was centrifuged (330×g for 5 min) to collect the cells, washed once more with PBS, and then the supernatant was removed before adding the culture medium. After cell counting, the cells were used for spheroid production.

The cHep was thawed according to the protocol recommended by the source of purchase (Thermo Fisher Scientific). The cHep that had been cryopreserved in body nitrogen was placed in a thermostatic bath at 37 °C, thawed to the extent that a thin icicle remained, and decanted into PBS. The cHep was centrifuged at 65×g for 4 min, and the medium was added after removing the supernatant. After cell counting, the cells were used for spheroid production. After cell counting, cHep was seeded with the cell number 5×10^3 /well. For two-mix groups, cHep with 5×10^3 cells and cASC with 1.5×10^3 cells/well were seeded for cHep + cASCs group and cHep with 5×10^3 and HUVEC with 3.5×10^3 cells/well were seeded for cHep + HUVEC group. For three-mix group (cHep + cASCs + HUVEC), cHep, cASCs, and HUVECs were mixed in a pipetting reservoir (AS ONE, Osaka, Japan) and spheroid culture medium was added. According to the protocol of previous studies [23,25], spheroids were cultured by mixing cells in the ratio of cHep: HUVEC: cASCs = 10: 7: 3 with the cell numbers, 5×10^3 , 3.5×10^3 , and 1.5×10^3 cells/well respectively. These cell mixtures were seeded in 96-well plates (96-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate; Corning, New York, USA) and prepared with HGM, EGM-2, and MSCGM in a ratio of HGM: EGM-2: MSCGM = 3: 1: 1, and recombinant ferine hepatocyte growth factor (rf-HGF) was added at a concentration of 10 ng/mL. The day of seeding was designated as day 0. The plates were mixed by pipetting and centrifuged at 100×g for 210 s every 12 h until day 2. Subsequently, several cell clumps were observed at the bottom of the plate. Spheroid culture was continued until day 7, and half of the medium was changed every 3 days. These diameters were measured on day 4 and day 7, and the spheroid formation ratio was calculated each day.

2.3. Quantitative real-time polymerase chain reaction

Total RNA was extracted using a RiboPure™ RNA Purification Kit (Thermo Fisher Scientific). Real-time polymerase chain reaction (RT-PCR) was performed using a PrimeScript™ RT Reagent Kit (Takara Bio, Shiga, Japan). Quantitative RT-PCR was performed in duplicate using a Thermal Cycler Dice® Real Time

System II (Takara Bio) with a TB Green® Premix Ex Taq™ II (Takara Bio) according to the manufacturer's instructions. After 3 min of denaturation at 95 °C, 40 cycles of two steps consisting of 5 s at 95 °C and 30 s at 65 °C or 60 °C were conducted. Real-time PCR reactions were performed using the Thermal Cycler Dice® Real Time System II (Takara Bio). Comparative quantification was performed for *albumin* (*ALB*), *α1-antitrypsin* (*α1-AT*), and *tyrosine aminotransferase* (*TAT*), which are markers of mature hepatocytes; for *α-fetoprotein* (*AFP*), which is a marker of immature hepatocytes; for *E-cadherin* (*CDH1*), which is a cell adhesion factor; and for *CYP2E1* and *CYP3A12*, which are drug-metabolizing enzymes. *β-actin* was used as an endogenous control. The primer sequences and temperature settings for the second step are listed in Table 1.

2.4. Histological evaluation and immunohistochemistry

The medium was washed three times with PBS to thoroughly remove the medium from the spheroids. The spheroids were fixed with neutral buffered 10% formalin for 10 min and embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E) staining and immunohistochemical staining for vimentin, ALB, and von Willebrand Factor (vWF). Blocking was carried out by adding PBS containing 5% skim milk, and immunohistochemistry was carried out by incubation overnight at 4 °C with polyclonal goat anti-dog albumin antibody (Bethyl Laboratories Inc., TX, USA), monoclonal mouse anti-vimentin antibody (clone V9; Agilent Technologies, California, USA), or polyclonal rabbit anti-human vWF antibody, HRP conjugated (Agilent Technologies). The corresponding secondary antibodies for albumin and vimentin included anti-goat antibody (Histofine Simple Stain MAX-PO(G), NICHIREI BIOSCIENCE, Tokyo, Japan) and anti-mouse antibody (Histofine Simple Stain MAX-PO(M), NICHIREI) for 30 min. As a negative control, 2 μg/mL normal goat IgG (R&D Systems, Minnesota, USA) and 1 μg/mL normal mouse IgG (Merck KGaA, Darmstadt, Germany) was used. All antibodies were diluted using an antibody diluent with background reducing components (Agilent Technologies). As color reaction, histofine SAB-PO(M) kit (NICHIREI) containing DAB (3,3'-diaminobenzidine tetrahydrochloride) and H₂O₂ was used.

3. Results

3.1. Spheroid formation

Cells were dispersed in the cHep and two-mix groups (cHep + cASCs and cHep + HUVEC), and small aggregates of cells started to form among dispersed cells in the three-mix group (cHep + cASCs + HUVEC) on day 1 (Fig. 1). Small aggregates of cells started to form on day 2 in the cHep and two-mix groups. Small aggregates of cells gradually increased in size in all groups; however, single large spherical cellular aggregates were formed only in the three-mix group, and multiple small aggregates or large but distorted shaped cells formed in the cHep or two-mix groups, respectively, on day 4. The diameter of the spheroids was approximately 500 μm in diameter on day 4 and slightly increased on day 7 in the three-mix group. The diameter of the three-mix group was significantly larger than that of the cHep group, with diameters of 516.7 μm and 438.5 μm, respectively. The ratio of spheroid formation evaluated on day 4 was significantly higher in the three-mix group (60.2%) than that in the two-mix group of cHep + cASCs (36.2%), cHep + HUVEC (24.6%), and cHep (5.9%) groups (Fig. 2). Thus, the three-mix condition was considered to be the best condition for forming large 500-μm spheroids.

Table 1
Sequence of PCR primers and condition of amplification.

gene	F/R	sequence (5'→3')	product size (bp)	Tm (°C)	
					2nd step
β -actin	F	GATGAGGCCAGAGCAAGAG	77	66.3	65
	R	TCGTCCCAGTTGGTGACGAT		67.9	
Albumin	F	GTTCTGGGCACGTTTTGTATGA	278	69.4	60
	R	CTTGGGGTGCTTTCTGGTGAAC		68.3	
AFP	F	CTTTGGCTGCCACTATGGC	182	68.8	60
	R	AACCGTTATGGCTCGGAAGGT		67.6	
α 1-AT	F	GCTGTCCAGGAGACGGATGAT	157	67.7	60
	R	CAAAGGCTGTAGCGATGCTCA		67.5	
TAT	F	TTCTGGCTGTGGCTGCAAGG	181	71	60
	R	AGGATCCAGCCCAACTCCA		70.2	
CDH1	F	GGTCTCACATTTCCAGTT	100	63.9	60
	R	AAATGGGCCTTCTCGTTT		62.3	
CYP2E1	F	CAGGACACGATGTCAGAGGA	116	65.3	60
	R	CTGGCTTGAACCTCTCGGATC		64.1	
CYP3A12	F	AAGGACTTCCTTTGTTCTCAAGAAA	86	65.8	60
	R	CCTACATGAGTGAACCACATAATCAA		65.5	

3.2. Hematoxylin and eosin and immunohistochemistry

The cellular composition of spheroids in the three-mix groups was assessed histologically. Hematoxylin and eosin-stained cross sections of three-mix spheroids composed of multiple small groups of cohesively attached polyhedral epithelial cells surrounded by several layers of spindle cell layers (Fig. 3). The polyhedral cells were immunohistochemically pancytokeratin-positive, and the spindle cells were positive for vimentin. vWF revealed HUVEC were present individually within spheroids. Rare vascular structure was also constructed (Supplemental data).

Pancytokeratin-positive cells were also positive for ALB. Comparing the results of the H & E and ALB staining, the region where the nucleus was lost due to acidophilic staining in the H & E

staining and the region where ALB protein was expressed by immunochemistry overlapped, indicating that cell death occurred.

3.3. Gene expression of hepatocyte related genes by quantitative PCR

Albumin expression levels in all groups, including the cHep, two-mix, and three-mix groups showed approximately the same level on days 4 and 7, which represent maintenance of mature hepatocytes (Fig. 4). Regarding the gene expression of AFP as a marker of hepatocyte progenitor cells, there was an increasing tendency from day 4 to day 7 in all cHep, two-mix, and three-mix groups, with the highest increase rate in the three-mix group, although this was not statistically significant. In addition, it was not statistically significant, but the expression level of AFP was highest in the three-mix group, followed by the two-mix groups, and that of cHep was the lowest among the four groups.

CYP2E1 and CYP3A12, as drug-metabolizing enzymes, showed an increasing tendency from day 4 to day 7 in all the cHep, two-mix, and three-mix groups, although the difference was not statistically significant (Fig. 4). CYP3A12 expression was lowest in the three-mix group on day 4, but highest on day 7, which represented the highest increase rate of CYP3A12 expression in the three-mix group. The expression levels of CYP2E1 and CYP3A12 among the four groups were not significantly different on days 4 and 7. The gene expression of other hepatocyte markers, including those of α 1-AT, and TAT and CDH1, in all the groups increased from days 4–7, indicating the maintenance of hepatic nature in all the groups (Supplementary).

4. Discussion

In this study, large canine hepatocyte spheroids (LcHS) with liver function was established by mixing and culturing with cHep, HUVECs, and cASCs using a low absorption plate. To the best of our

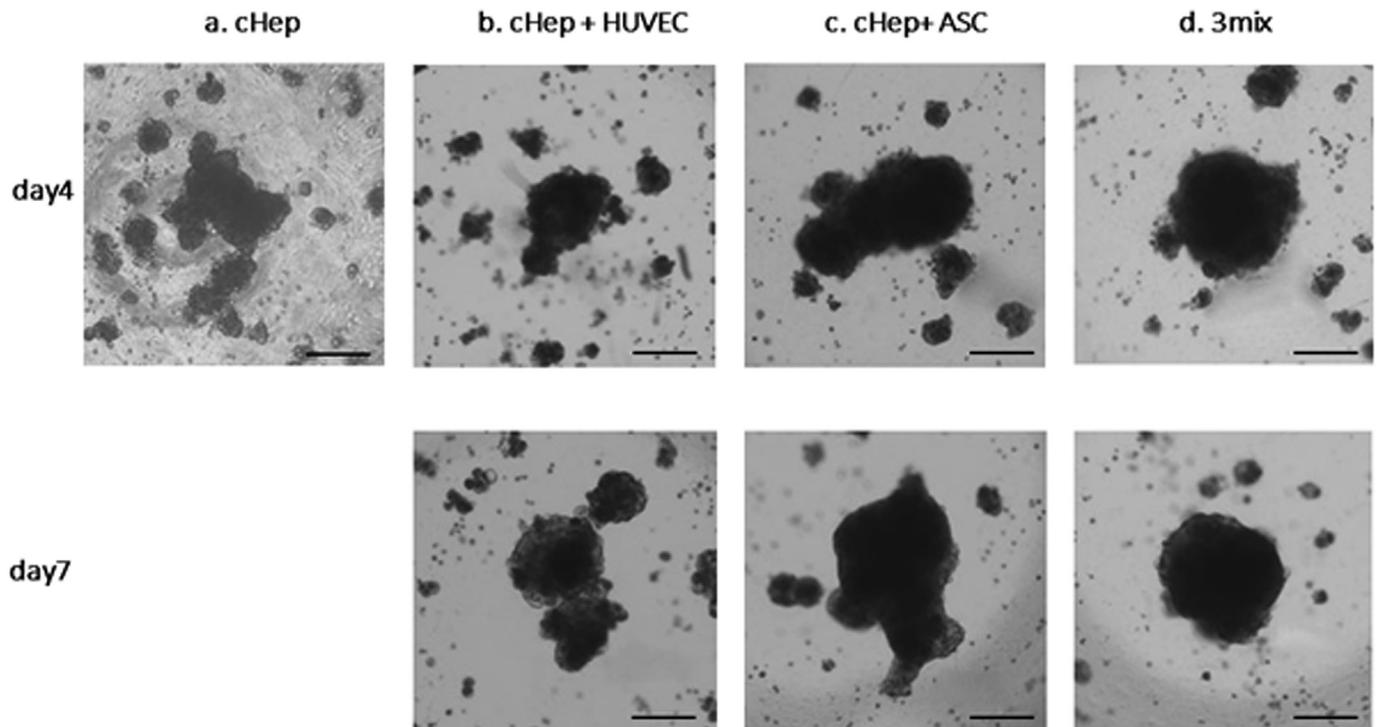


Fig. 1. Inverted microscope findings of spheroids. Morphological findings of spheroids included a; Hepatocyte, b; Hepatocyte + HUVEC, c; Hepatocyte + ASC, and d; 3mix on day 4 and 7. In the cell groups a, b, and c incomplete round spheroids were observed and the small aggregation of cells were scattered around. In the 3 mix group, the cells clumped into a sphere and round spheroid was observed. The morphology of each spheroid was similar on days 4 and 7. The bar is indicated 300 μ m.

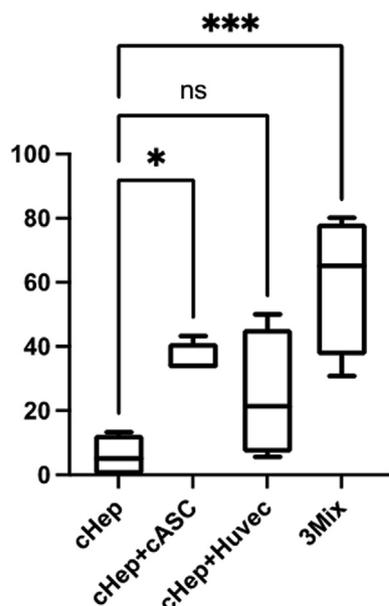


Fig. 2. Comparison of spheroid formation ratio on day 4: Spheroid formation is significantly higher in cHep + cASC and 3 mix group than cHep group. (One way anova test followed by Dunnett's multiple comparison test; * $P < 0.05$, *** $P < 0.001$).

knowledge, there has been no report of producing canine hepatocyte spheroids in mixed culture, and this is the first report of the production of LcHS by three-mix cultures. Here, we indicated that LcHS enables easy and efficient large-scale hepatocyte culture in dogs and suggests that LcHS can be applied in the development of toxicity evaluation tools and transplantation medicine as a basic technique. In liver regenerative medicine research, many studies have been conducted on the formation of spheroids and their culture improvement in mice, humans, and dogs [11–14]. In humans, studies have been conducted in vitro to culture tissues that are physiologically and functionally closer to in vivo organs by producing spheroids by mixing multiple cell types. Yanagi et al. demonstrated that ASCs promoted hepatocyte aggregation and affected the formation of spheroids with sufficient structural strength and appropriate shape [24,26]. Furthermore, transplantation of human hepatocyte spheroids into mice induced

angiogenesis and albumin production, indicating that they exerted liver function even in heterogeneous liver transplants [24]. In addition, it was predicted that spheroids made from human hepatocytes maintained cytochrome p450 enzyme expression and activity for 21 days, within three times the observed value of the intrinsic clearance of the test compound [27]. Van Pel et al. reported that these spheroids can be laminated to achieve an even greater organization using 3D bioprinting technology [28]. When some types of plate were applied for spheroid formation in preliminary studies, we found large differences in spheroid formation ratio and shape, cell death rate, and albumin expression. Furthermore, canine induced hepatocyte spheroids showed the high expression of hepatic genes, such as *albumin* and drug metabolism *CYP* genes [11]. Finally, it was suggested that a larger canine liver organization could be realized using LcHS.

In this study, it was revealed that the ratio of spheroid formation was significantly improved by the three-mix culture compared to cHep alone or the two-mix, which were cHep + cASCs and cHep + HUVECs, respectively. On day 4, the spheroid formation rate was the highest (76.7%) in the three-mix culture, which was more than six times higher than that of cHep alone (11.9%). Some studies in other animal species have demonstrated that mesenchymal stem cells and vascular endothelial cells promote liver regeneration. Matsumoto et al. reported that the presence of mesenchymal cells and vascular endothelial cells may be involved in the morphogenesis that occurs during liver development, even before angioplasty [29]. Furthermore, the cellular environment of liver tissue development is reproduced and hepatocytes can be self-assembled when specific human hepatocytes, mesenchymal stem cells, and vascular endothelial cells are co-cultured [25], which occurs by reproducing the cell–cell interaction of the cells and forming 3D liver buds [25]. In addition, it has been reported that the function of MSCs to promote hepatocyte aggregation has an effect [24,26]. We consider that cASCs and vascular endothelial cells may play an auxiliary role in the organization of canine hepatocytes, similar to the mechanism of other species. Thus, it was suggested that even in dogs, a mixed culture of not only hepatocytes but also other cASCs and vascular endothelial cells contributes to the improvement of proliferation efficiency and function.

Many factors such as cell mixing ratio, plate, medium, cell distribution within spheroids, pipetting and plate centrifugation, and hepatocyte growth factor (HGF) are important for the production of

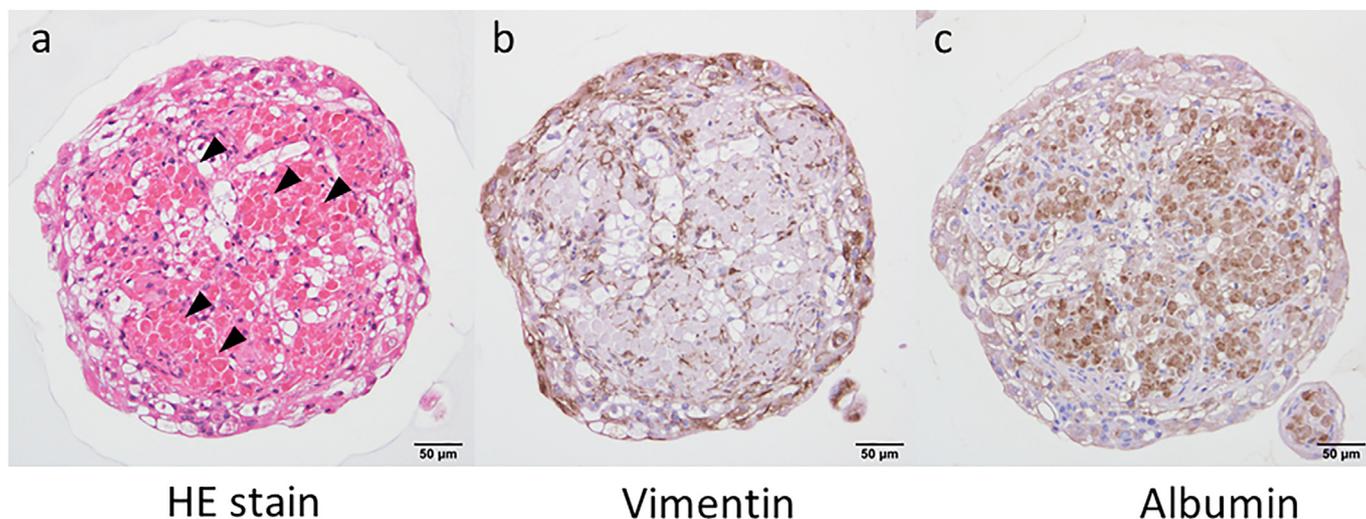


Fig. 3. Histopathological findings and immunohistochemistry of spheroids. a H&E stain, b Vimentin, c Albumin. a; cHep is distributed in the center of spheroids. b; Vimentin-positive cells are predominantly located on the peripheral of the spheroid, and vimentin-positive region were identical to the area of HUVEC and cASCs. c; Albumin-positive cells were located inside the spheroids, indicating that cHep was distributed in the central part. Areas of cell death are indicated by arrowheads.

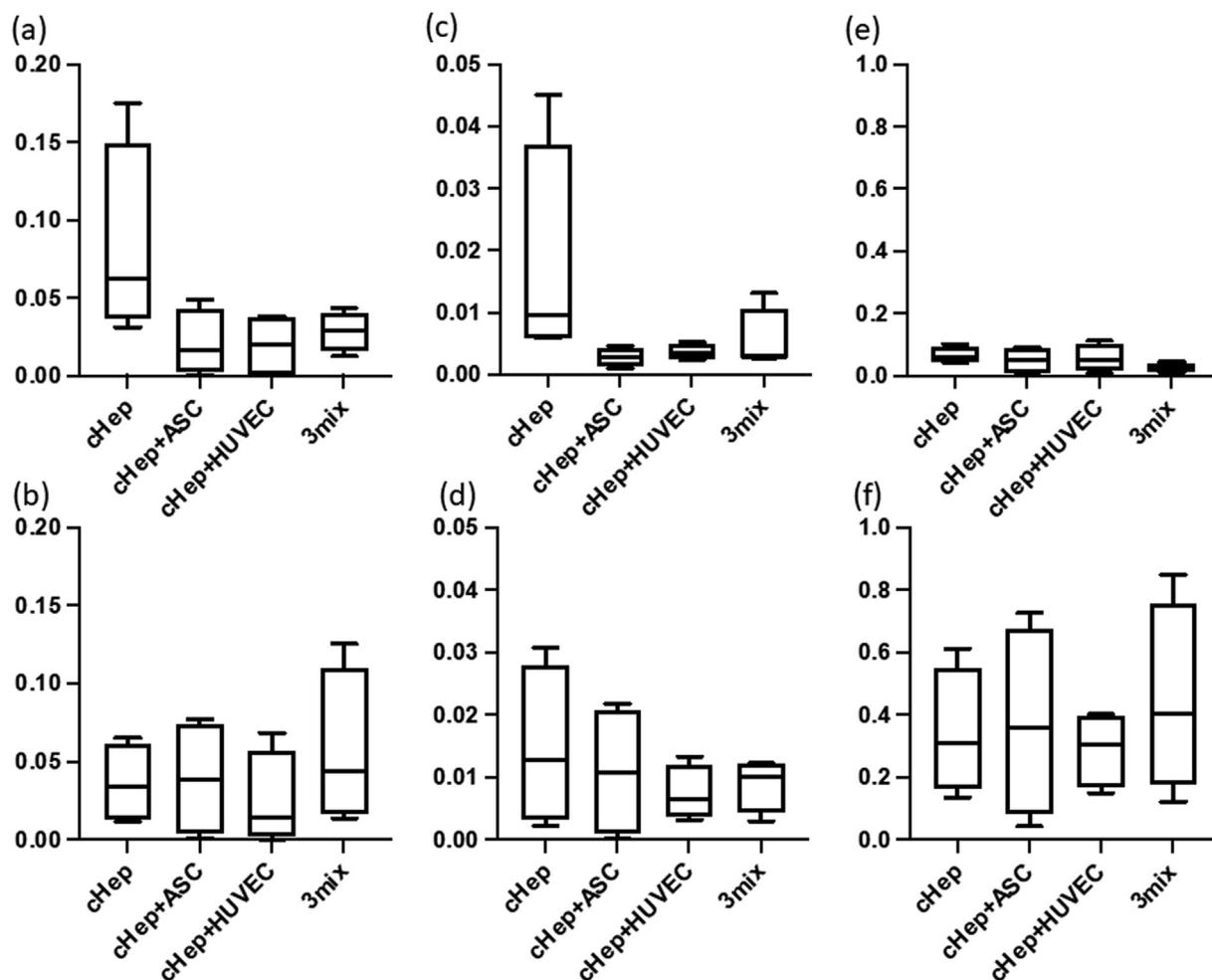


Fig. 4. Albumin and Cyp m-RNA expression levels in all groups, including the cHep, two-mix, and three-mix groups on days 4 and 7. a: Albumin expression on day 4, b: Albumin expression on day 7, c: CYP2E1 expression on day 4, d: CYP2E1 expression on day 7, e: CYP3A12 expression on day 4, and f: CYP3A12 expression on day 7. Albumin, Cyp2E1 and Cyp3A12 expression levels was not changed in each group on day 4 and 7, respectively (One way anova test followed by Tukey's multiple comparison test). Compared with Day 4 and 7, Cyp3A12 levels of all groups on day 7 were tend to increase than on day 4, but the differences were not significant (unpaired student t test).

spheroids that maintain liver function [18,24], which have also been shown to be involved in other reports. In this study, the cell mixing ratio was cHep: HUVEC: cASCs = 10: 7: 3, given that the mixing ratio of these three cells at various ratios were preliminarily examined and other cell ratios did not form spheroids of appropriate size and shape, and severe hepatocyte cell death occurred (data not shown). In other preliminary study, the spheroid formation ratio did not change in either group between the medium for hepatocytes and the medium for three-mix groups. When we compared the condition for hepatocytes or three-mix groups with the culture medium containing solely hepatocyte medium or hepatocyte medium with HUVEC and cASC medium. Spheroid formation was not found in hepatocyte group in either culture medium, which precluded the medium's affection for the low spheroid formation of hepatocyte group (data not shown).

The character of the plate was also important, as the three-mix cultures did not fall to the bottom of the U-bottom as well as in other plates, inhibiting spheroid formation. Therefore, in this study, spheroids were prepared using Corning 96-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate (Corning), which is characterized by low adhesiveness. In the previous study, the medium used for spheroid culture was William E medium, mainly in the human hepatocytes [25], but there was no culture of canine spheroids. Therefore, the mixed medium including

hepatocyte medium, HUVEC medium (EGM-2, Lonza), and cASC medium (MSCGM, Lonza) were mixed at a ratio of 3: 1: 1 and used for spheroid culture, and appropriate culture conditions were determined. We suggest that these culture conditions are optimal for the production of LChS.

The three-mix spheroids produced in this study maintained the expression of liver-related genes equivalent to those of cHep alone and the two-mix group. Gene expression of liver-related genes *ALB*, *α1-AT*, *TAT*, *AFP*, *CDH1*, *CYP2E1*, and *CYP3A12* were assessed by quantitative PCR. According to the results of this gene expression analysis, the gene expression of the three-mix spheroids was similar to that of cHep alone on day 7 (Fig. 4). However, the expression level of liver-related genes in the three-mix spheroids was only 30% compared with cHep alone on day 0. The reason for this result is that the ratio of hepatocyte cell construct in the three-mix cultures on day 0 was only 50%, indicating that a small volume of hepatocytes will affect low liver-related gene expression in the three-mix cultures. In this study, when performing quantitative PCR, the same amount of mRNA obtained from all cultured pattern spheroids was used for PCR. Therefore, in the case of the two- and three-mix cultures, it is highly possible that the amount of hepatocyte-derived mRNA is smaller than that of hepatocytes alone due to the inclusion of cASCs and HUVEC mRNA. The expression level of liver-related genes in the mixed culture was overestimated compared to cHep

alone. In humans, it has been reported that liver function genes such as *ALB* are significantly increased in the three-mix culture, which is what differs from our research results [25]. In dogs, the presence of stimulators, growth factors, and cytokines from stem cells that differ from humans should be considered. Unfortunately, it has been unclear why hepatocyte characters are different between dogs and other animals, since there are few reports on the characteristics of hepatic stem cells involved in liver regeneration and development in dogs. Then, the factors involved in regeneration and cell differentiation are still unknown. However, our previous reports demonstrated that albumin production, *CYP* expression, urea production ability, etc. are generally low even if hepatocyte induction is performed using the same protocol as in humans and rats [11]. Perhaps factors different from those of humans and mice are important for canine hepatocyte differentiation.

Interestingly, in the three-mix spheroids, hepatocyte populations were distributed inside the spheroid, and cASCs were mainly distributed around the spheroid surrounding the hepatocyte population. HUVEC was present within the spheroids. In humans and mice, hepatocytes have been reported to be distributed in the spheroid margin, and the cell distribution was significantly different in this study [24]. Cell death occurred inside the spheroid. Multiple factors are considered, including large spheroid diameter [30,31], quality of hepatocyte, medium condition, and additive factors. The central part becomes hypoxic, and cell death occurs [30]; consequently, a large spheroid with a diameter of more than 100 μm is produced in the liver. Therefore, it has been reported that the spheroid diameter should be limited to within 200 μm [31,32]. The location of the HUVEC with constructing vascular-like structure within the spheroid may be a good condition for hepatocytes to receive oxygen. Another potential function of HUVEC is cytokines production including IL 6, cytokine for the hepatocyte proliferation [33]. Further investigation with increased HUVEC cell number is required to expect more appropriate environment for hepatocytes to survive for long term with function. In addition, the MSCs are expected to initiate condensation of the spheroids cell mixtures, suggesting optimal mechanical properties are promoted for the formation of 3D. MSC-based traction force produced by the actomyosin cytoskeletal axis plays an important role in the directed and drastic movements of cell collectives [34]. However, the role of canine actomyosin cytoskeletal axis is unclear, the function should be investigated in future. To increase the survival rate of hepatocytes, it is necessary to elucidate the factors of cell death inside spheroids and improve the culture protocol. We suggest that cell death and the inside population of hepatocytes in the three-mix spheroid might prevent the expression of liver-related genes.

Another important significance of this study is the production of spheroids with a diameter of about 500 μm in the early days of culture, which are suitable for lamination using a 3D bioprinter (Regenova®, Cyfuse, Tokyo, Japan). The 3D bioprinter Regenova® holds spheroids with sword-shaped needles lined up at 400 μm intervals. In addition, Yanagi Y et al. recommended that stacking with a 3D printer be performed with spheroids around day 3 [24]. In this study, three-mix spheroids formed 76.7% of spheroids, compared with 11.9% cHep alone on day 4, indicating that three-mix spheroids could be applied to produce liver tissue using a 3D bioprinter in the future. In our study, cHep tends to be located in the center of the spheroid and surrounded by mesenchymal cells. This indicates disadvantage for the hepatocytes to obtain growth factors or cytokines in the culture medium. Spheroid with 500 μm in diameter was chosen for the purpose of constructing 3D structures using a 3D printer (Regenova). Perhaps, 3D structure increase cellular attachment which is favorable for the hepatocytes to express the function [24]. In future, 3D printing technology using smaller spheroids can be desired.

This study has some limitations. First, the three-mix spheroids did not improve the expression of liver-related genes such as *ALB* and *CYP*, and it is necessary to search for the optimal combination of culture equipment for dogs. Second, the cell distribution is different from that of other species, and hepatocyte death inside the spheroid occurs; thus, the cause and improvement should be investigated. Third, although we used canine MSCs and HUVECs, we should improve the culture method, that is, for the iPS cells and other artificial blood vessels. Fourth, canine albumin production by the cHeps are perfectly evaluated by measuring albumin secretion in the culture supernatant, however FBS within the culture medium precludes the measurement of canine albumin secretion. Then, distinct assay system to detect canine albumin production should be established.

In this experiment, we succeeded in producing LcHS with a high spheroid formation ratio, and LcHS maintained liver function similar to hepatocyte-alone spheroids. The present study indicates the potential of this LcHS for immediate stacking in 3D bioprinters. Findings from this study are expected to be applied in drug toxicity tests for drug discovery research and transplantation medicine for the treatment of canine liver diseases in the future. Comprehensive genetic analysis and toxicity tests are highly needed for further investigation.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2021.11.007>.

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