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Cold storage of porcine hepatocyte spheroids for spheroid bioartificial liver

Yi Li^{1,2,3}, Harvey S. Chen^{2,3}, Mohammed Shaheen^{2,3}, Dong Jin Joo^{2,3}, Bruce P. Amiot², Piero Rinaldo⁴, Scott L. Nyberg^{2,3}

¹Laboratory of Pathology, Key Laboratory of Transplant Engineering and Immunology, NHFPC, West China Hospital, Sichuan University, Chengdu, China

²Department of Surgery, Mayo Clinic, Rochester, Minnesota

³William J. von Liebig Center for Transplantation and Clinical Regeneration, Mayo Clinic, Rochester, Minnesota

⁴Department Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota

Abstract

Background and aims: Cell-based therapies for liver disease such as bioartificial liver rely on a large quantity and high quality of hepatocytes. Cold storage was previously shown to be a better way to preserve the viability and functionality of hepatocytes during transportation rather than freezing, but this was only proved at a lower density of rat hepatocytes spheroids. The purpose of this study was to optimize conditions for cold storage of high density of primary porcine hepatocyte spheroids.

Methods: Porcine hepatocytes were isolated by a three-step perfusion method; hepatocyte spheroids were formed by a 24 hours rocked culture technique. Hepatocyte cell density was 5×10^6 /mL in 1000 mL spheroid forming medium. Spheroids were then maintained in rocked culture at 37°C (control condition) or cold stored at 4°C for 24, 48 or 72 hours in four different cold storage solutions: histidine-tryptophan-ketoglutarate (HTK) alone; HTK + 1 mM deferoxamine (DEF); HTK + 5 mM *N*-acetyl-L-cysteine (NAC); and HTK + 1 mM DEF + 5 mM NAC. The viability, ammonia clearance, albumin production, gene expression, and functional activity of cytochrome P450 enzymes were measured after recovery from the cold storage.

Results: In this study, we observed that cold-induced injury was reduced by the addition of the iron chelator. Viability of HTK + DEF group hepatocyte spheroids was increased compared with other cold storage groups ($P < 0.05$). Performance metrics of porcine hepatocyte spheroids cold stored for 24 hours were similar to those in control conditions. The hepatocyte spheroids in control conditions started to lose their ability to clear ammonia while production of albumin was still active at 48 and 72 hours ($P < 0.05$). In contrast, the viability and functionality of hepatocyte

Correspondence: Scott L. Nyberg, William J. von Liebig Center for Transplantation and Clinical Regeneration, Mayo Clinic, Rochester, MN. Nyberg.scott@mayo.edu.

CONFLICT OF INTERESTS

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spheroids including ammonia clearance and albumin secretion were preserved in HTK + DEF group at both 48- and 72-hour time points ($P < 0.05$).

Conclusions: The beneficial effects of HTK supplemented with DEF were more obvious after cold storage of high density of porcine hepatocyte spheroids for 72 hours. The porcine hepatocyte spheroids were above the cutoff criteria for use in a spheroid-based bioartificial liver.

Keywords

cold storage; hepatocyte; porcine; spheroid

1 | INTRODUCTION

Cell therapies including liver support therapy, tissue engineering liver transplantation, and hepatocyte transplantation have emerged as potential treatments of liver failure, in response to the shortage of transplantable organs.¹ Primary hepatocytes are the first and most obvious candidate for use in cell therapy.² They also have been used for many aspects of biochemical and pharmaceutical research to understand the biological processes occurring in the liver. However, they do not survive long³ or proliferate⁴ in vitro and easily lose their expression of liver-specific genes.⁵ Although human hepatocytes are an ideal source, shortage of donor livers available for hepatocyte isolation is the limiting factor. Porcine hepatocytes are a promising alternative to human hepatocytes for their functional similarity. Devices like bioartificial liver require a sufficiently large quantity of metabolically active, high-quality porcine hepatocytes, usually up to 2.5×10^{10} (200 g) hepatocytes. Such a large quantity of highly viable and functional cells may not be feasible using fresh isolation techniques for each treatment. In addition, transportation of freshly isolated porcine hepatocytes to remote sites for clinical use is often not possible, which makes it hard to develop a treatment network of these liver support systems.

The HepatAssist device, a first-generation liver support system, utilized frozen porcine hepatocytes as cell resource to treat acute liver failure in randomized clinical trials.⁶ However, hepatocytes of the HepatAssist system were susceptible to freeze-thaw damage. Freezing and rewarming resulted in a loss of viability and functionality, as well as release of cellular components and an associated immune response.⁷ The lesions were amplified as cold preservation time was prolonged. As a result, improved survival after treatment with the HepatAssist device was only observed in patients with fulminant/subfulminant hepatic failure and the limited efficacy of the device was likely related to the reduced functionality of cryopreserved hepatocytes after thawing. An important lesson from the HepatAssist trial was that hepatocytes must avoid injury and remain viable during the transport process to retain their differentiated functions during a subsequent treatment session. We hypothesize that cold storage cryopreservation of hepatocytes at a chilled but non-frozen temperature may offer a potential solution for transporting hepatocytes to distant sights and developing treatment networks for bioartificial liver support systems.

Hepatocytes are an anchorage-dependent epithelial cell type that are susceptible to a form of apoptotic cell death, termed anoikis, after isolation and separation from their basement membrane.⁸ However, anoikis of hepatocytes may be prevented by their formation into

three-dimensional tissue structures of multi-cell cell aggregates termed hepatocyte spheroids.⁹ Hepatocyte spheroids may be formed most rapidly under rocker suspension culture conditions.¹⁰ Reports of high viability, high levels of liver-specific functions, polarity, and bile canaliculi formation by primary hepatocyte spheroids provide further evidence that spherical geometry mimics the hepatocellular micro structure of the liver.¹¹ These features allow hepatocyte spheroids to serve as an alternative geometric shape of liver tissue for ex vivo research and therapeutic applications. Porcine hepatocytes are a reasonable alternative to human hepatocytes because the metabolic profiles of porcine hepatocytes are basically similar to those of humans. Genetically engineered fumarylacetoacetate hydrolase (FAH)-deficient pigs as incubators for in vivo large-scale expansion of primary human hepatocytes are promising option for future demands.¹²

To date, the cold storage of primary porcine hepatocyte spheroids is poorly studied. It is known that cold-induced injury of hepatocytes increases the intracellular chelatable “redox-active” iron, catalyzing the Haber-Weiss reaction to generate cytotoxic reactive oxygen species (ROS), such as hydroxyl (OH^-), superoxide (O_2^-), and hydrogen peroxide (H_2O_2).¹³ Lipid peroxidation, mitochondrial permeability transition, and damages of cellular macromolecules subsequently increase owing to the strong oxidizing action of free radicals. These injuries could be inhibited by supplementation of iron chelators to the preservation solution. Deferoxamine (DEF), an agent to treat iron accumulation, can lower the formation of the free radicals by binding Fe^{3+} ions to form complexes, thus preventing cell death.¹⁴ N-acetyl-L-cysteine (NAC), a thiol and major precursor of L-cysteine, protects the liver by increasing hepatic glutathione (GSH).^{15,16} It combines with foreign substances and neutralizes free radicals and reactive oxygen compounds. NAC is widely used to treat paracetamol (acetaminophen) overdose.

Therefore, our experimentation was aimed at examining the effect of cold storage treatment on the viability and liver-specific functionality including albumin secretion and ammonia clearance of porcine hepatocyte spheroids. We evaluated the beneficial effects of DEF and NAC in preservation solution HTK. Along with optimizing conditions for cold storage of hepatocyte spheroids, a second purpose of this study was to determine the duration of safe cold storage in terms of integrity and functionality of spheroids under these conditions. Answers to these questions are needed to establish foundations to develop liver support devices, other cell-based therapies, and treatment networks for these future therapies.

2 | MATERIALS AND METHODS

2.1 | Primary hepatocytes isolation

All animal procedures were performed in accordance with the guidelines set forth by the Mayo Institutional Animal Care and Use Committee and the National Institutes of Health with the approvals.

The timeline of the experimentation is shown in the Figure 1. Wild-type male large white swine was obtained from a local vendor (Manthei Hog Farm, Elk River, MN). Donor animals were started on 40% protein diet 7 days prior to harvest. Animals were anesthetized with 5 mg/kg telazol, 2 mg/kg xylazine, 0.31 mg/kg buprenorphine and 0.01 mg/kg

glycopyrrolate, and maintained with inhaled 1%–2% isoflurane. A midline laparotomy from xiphoid to umbilicus was performed; the hepatic artery and the bile duct were then ligated. Heparin sodium at the dose of 200 U/kg body weight was injected into the infrahepatic vena cava. Hepatocytes were isolated from a donor animal using a modified three-step collagenase perfusion method as previously described.¹⁷ Five minutes later, the portal vein was cannulated. The liver was then infused with 7 L of perfusate I (NaCl 8.3 g/L, KCl 0.5 g/L, HEPES 2.4 g/L, EGTA 0.95 g/L, NAC 0.8 g/L) at rate of 800 mL/min. The inferior vena cava was dissected as outflux. The liver was then excised and infused with 2 L of perfusate II (NaCl 8.3 g/L, KCl 0.5 g/L, HEPES 2.4 g/L, NAC 0.8 g/L) in a sealed container at the same speed. The animals were then euthanized. After suctioning away all the liquids, 2 L of perfusate III (NaCl 3.9 g/L, KCl 0.5 g/L, HEPES 2.4 g/L, CaCl₂·2H₂O 0.07 g/L, NAC 0.8 g/L, bovine serum albumin 4 g/L, and Liberase 130 Wunsch U/L; Serva, KG, Germany) was added at 37°C, and the solution was subjected to 30–35 min of recycle perfusion at a rate of 1200 mL/min. The collected cell suspension was washed three times with wash media (Williams' Medium E 10.8 g/L, NaHCO₃ 2.2 g/L, HEPES 2.6 g/L, streptomycin 0.1 g/L, and penicillin 100 000 U/L) and centrifuged at 4°C for 10 minutes at 56×g.

To eliminate experimental bias, all harvested hepatocytes with yield more than 100 g or viability exceeding 90% by trypan blue dye exclusion were brought to further experimentation.

Freshly isolated hepatocytes were suspended in spheroid forming medium (SFM) composed of 10.8 g/L William's E supplemented with 2.2 g/L NaHCO₃, 200 U/L insulin, streptomycin 0.1 g/L, and penicillin 100 000 U/L, 10% vol/vol fetal bovine serum, and 1000 U/L heparin. The cells were inoculated into four 1500 mL spheroid generation chambers at a concentration of 5×10^6 viable cells/mL in 1000 mL of media per chamber, as described previously.^{17,18} Spheroid chambers were incubated in a humidified incubator at 37°C with a 5% CO₂ atmosphere and rocked continuously at a frequency of eight cycles (0.12 Hz) for 24 hours to induce spheroid formation.

2.2 | Cold storage and recovery

Spheroids were collected, washed, and enriched after centrifugation, followed by re-suspending using 125 mL cold storage medium at the density of 4×10^7 cells/mL (HTK alone; HTK + 1 mM DEF; HTK + 5 mM NAC; HTK + 1 mM DEF + 5 mM NAC). The spheroid suspensions were then transferred into infusion bags (Intravia Container, Baxter Healthcare), respectively, and placed in a refrigerator at 4°C to be cold stored. Another 5×10^9 spheroids were left in rocked culture without cold storage as control. Cold storage experiments were performed in each isolation (N = 10).

After 24, 48, and 72 hours of cold storage, 2×10^7 spheroids were sampled and centrifuged at $50 \times g$ for 5 minutes, and the supernatant fluid was removed. Diluted spheroid suspensions in 20 mL SFM containing 2.5% vol/vol heavy deuterium-enriched ammonia gas (Cambridge Isotope Laboratories Inc, Andover, MA) were rocked in glass dishes (10 × 8 × 2 cm) custom-made by Mayo Division of Engineering and pre-siliconized with Sigmacote and cultured.

Representative samples were removed from the spheroid dishes to determine the spheroid number, diameter, and total volume (cell mass) by using Multisizer 3 (560 μm aperture; Beckman Coulter). Viability of hepatocyte spheroids was evaluated using the Fluoroquench™ viability stain (One Lambda, Canoga Park, CA) by inverted epifluorescence microscopy (Axioscope, Carl Zeiss, Inc, Thornwood, NY). Supernatant medium and spheroids were collected and stored at -20°C prior to analysis.

2.3 | Evaluation of hepatocyte spheroids

Evaluations were performed after cold storage of 24, 48, and 72 hours and another 24-hour recovery (day 3, day 4, and day 5, respectively).

Concentrations of heavy ammonia ($^{15}\text{ND}_3$) were quantified by capillary gas chromatography/mass spectrometry (GC/MS) as previously reported.^{11,19}

The concentration of pig albumin in medium was determined by enzyme-linked immunosorbent assay kit (Bethyl Labs, Montgomery, TX) according to the manufacturer's instructions.

Total RNA was extracted from spheroids at each time point using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reversely transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA), and qRT-PCR analysis was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). All assays were performed in triplicate. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an endogenous internal control, and the results were normalized by to the freshly isolated hepatocytes as $\text{Ct} = \text{Ct target gene} - \text{Ct GAPDH}$; $\text{Ct} = \text{Ct treated} - \text{Ct control}$, and fold = $2^{-\text{Ct}}$.

2.4 | Statistics

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a one-way ANOVA and Dunnett's test. A level of $P < 0.05$ was accepted as significant. All data were analyzed with SPSS software 17.0 version and organized with GraphPad Prism 5.

3 | RESULTS

3.1 | Viability of hepatocyte spheroids after cold storage

A total of ten donor animals with an average weight of 16.6 ± 1.5 kg were sacrificed for liver harvest and spheroid formation. Average harvest yielded 192.6 ± 13.2 g of primary hepatocytes per pig, with an average hepatocyte viability of $95.3 \pm 0.6\%$. Light microscopic examinations of the fresh single cell suspension and 24-hour culture suspension indicate the success of hepatocyte isolation (Figure 2A) and high quality of spheroid formation (Figure 2B). The spheroid number, diameter, and total volume of the spheroids were statistically comparable in each isolation before cold storage (Figure 2C–D).

Viability data using Fluoroquench™ staining showed progressively decreased cell viability and increased necrosis within spheroids after the cold storage (Figure 3A). On days 3 and 4 (after 24- and 48-hour cold storage), the viability was favorable in control group (97.3% and

96.4%), HTK + DEF group (94.3% and 94.6%), HTK + NAC group (92.8% and 95.1%), and HTK + DEF+NAC group (94.0% and 96.4%). The viability on day 5 was greater in the control group (97.7%), HTK + DEF group (92.8%), and HTK + DEF+NAC group (94.7%), which was higher than spheroids maintained under HTK (79.2%) and HTK + NAC (88.1%; $P < 0.05$). High density of hepatocyte spheroids can tolerate cold storage under the condition of HTK supplemented with DEF for up to 72 hours (Figure 3B).

3.2 | Albumin production and gene expression

Albumin production was greatest in the control group and had no significant difference among cold storage conditions of HTK group, HTK + DEF group, HTK + NAC, and HTK + DEF+NAC on days 3 and 4 ($P < 0.05$). On day 5, albumin production was similar in control group and HTK + DEF group. Albumin gene expression confirms the pattern of albumin production ($P < 0.05$) (Figure 4A–B).

Ammonia clearance of HTK + DEF group and HTK + DEF+NAC group was similar from day 3 to day 5. A clearance plateau occurs before rapid descent. The HTK group and HTK + NAC group had lower ammonia clearance capability ($P < 0.05$). Strikingly, all cold storage conditions were higher than spheroids in control group in ammonia clearance ($P < 0.05$; Figure 5A).

Expression of the six urea cycle genes including arginase 1 (Arg1), argininosuccinate synthase 1 (Ass1), argininosuccinate lyase (Asl), carbamoyl phosphate synthase 1 (Cps1), *N*-acetylglutamate synthase (Nags), and ornithine transcarbamylase (Otc) was detected to verify the ammonia clearance. Urea cycle gene expression levels decreased under all conditions from day 3 to day 5, compared to newly formed spheroids on day 2. The HTK + DEF group and HTK + DEF + NAC group had higher expression levels of urea cycle genes, compared to spheroids in HTK group and HTK + NAC group ($P < 0.05$). Similarly, spheroids in control group showed lower expression of urea cycle genes ($P < 0.05$; Figure 5B). We further examined the expression of liver-related genes including Cyp1a2, Cyp2e1, and hepatocyte nuclear factor 4 (Hnf4). These genes decreased under all cold storage conditions compared to control group, but were higher in both HTK + DEF group and HTK + DEF + NAC group compared to HTK group and HTK + NAC group ($P < 0.05$; Figure 5C).

According to the ammonia concentration, we calculated the slope of the clearance as

follows: Clearance slope = $\frac{C_{\text{Final}} - C_{\text{Initial}}}{t}$. On days 3 and 4, the ammonia clearance slope

was favorable in HTK + DEF group (-153.3 ± 7.1 and -140.8 ± 2.0), HTK + NAC group (-151.5 ± 7.1 and -139.4 ± 2.5), and HTK + DEF + NAC group (-151.2 ± 5.6 and -141.1 ± 2.9). The viability on day 5 was greater in HTK + DEF group (-133.7 ± 4.6) and HTK + DEF + NAC group (-132.4 ± 2.7), which was higher than spheroids maintained in control group (-96.8 ± 7.1) or under HTK (-112.2 ± 7.8) and HTK + NAC (-117.3 ± 7.0) cold storage conditions ($P < 0.05$; Figure 5D).

4 | DISCUSSION

Hepatic cold-induced injury remains a significant problem to the widespread implementation of cell therapies. It causes increased damage to the liver tissue and isolated hepatocytes by releasing of intracellular chelatable iron and formation of ROS.^{13,20,21} ROS then causes apoptosis and autophagy. The presence of iron chelators in storage media can protect organs and isolated hepatocytes from damage during cold storage and warm recovery. The HTK solution, a well-known preservation solution, is commonly used for donor heart, kidney, lung, and pancreas preservation in transplantation.²² Iron chelator, DEF, is often used to treat genetic or acquired iron accumulation. By the essential characteristic of binding iron, DEF is also widely used to protect oxidative stress and reduce the damage to various organs and tissues, such as the liver.²³ NAC is an antioxidant that provides protective effect to resist injury caused by ROS, being proven via regulation of the JNK/Bcl-2 pathway.²⁴

In our study, we observed viability of hepatocyte spheroids stored in HTK and the addition of DEF and/or NAC. It has been shown that HTK supplemented with DEF and/or NAC could inhibit the occurrence of apoptotic morphology in hepatocyte culture in first 2 days. When cold storage time was extended to 72 hours, there was an obvious decrease in viability following rewarming. None of cold storage conditions exceeded the continuous culture in control group. The liver is a major site of metabolism, synthesis, and detoxification, performing a complex and indispensable array of over 500 functions. Ammonia elimination is both a functional marker and a pathogenic molecule. Ammonia elimination correlates with ICP and neuroprotection in our previous large animal studies of SRBAL.^{25,26} Thus, ammonia clearance is a much more reliable marker of hepatocyte function. We observed the similar results in albumin production, but when it came to ammonia clearance, which was one of the most important biological characteristics, the results changed. The ammonia clearance and urea cycle genes detection in our data indicated similar rates by hepatocyte spheroids in cold storage solutions with additives for first 48 hours, followed by obvious decrease in the HTK + NAC group. Besides, ammonia detoxification and ureagenesis capacity of hepatocyte spheroids showed a more significant drop under control condition.

University of Wisconsin (UW) solution is the standard criterion for liver organ preservation, but its high levels of potassium and hydroxyethyl starch can perplex the additive fabrication, wash, and recovery procedures during cold storage. HTK is characterized by low osmolarity and electrolyte concentrations. HTK solution is easy to perfuse and flush for donor organs, and substances can be added to scavenge free radicals. The superior results of addition of NAC are likely due to histidine was partly combined with NAC to form *N*-acetyl-L-histidine, altering affinity for Fe²⁺, and redox activity of its iron complexes. Some researchers substituted histidine in HTK solution with *N*-acetyl-L-histidine in short-time heart preservation and proved effective.²⁸ In our experiment, under the protective condition, cold-induced apoptosis in HTK + NAC group was attenuated in hepatocyte spheroids, but this protection showed effective within two days. Thus, HTK supplemented with NAC is only suitable for cold storage within 48 hours, it may apply to instant transfer in hospitals or in near cities.

The presence of DEF during cold storage suggested chelation of redox-active iron can reduce cold-induced injury and decrease the subsequent vulnerability of isolated hepatocyte spheroids. Especially, ammonia removal and urea production are important considerations for cold storage of spheroid for use in cell-based therapy programs. The HTK supplemented with additive preservation solutions well retained albumin secretion and ammonia elimination that were easily to lose in continuous culture. Hepatocyte spheroids in HTK + DEF groups showed higher viability, lasting albumin production, and ammonia clearance. Addition NAC (HTK + DEF + NAC group) had extra protective effect at first 48 hours but with no statistical significance. The beneficial effects of DEF were more obvious after cold storage 72 hours compared to those of NAC only. The porcine hepatocyte spheroids stored in the combination of HTK + DEF for 72 hours were above the cutoff criteria for use in a spheroid-based bioartificial liver, which fulfill the demand of most of the worldwide transportation.

Based on a previous study, a similar effect of cold preservation of lower density and total amount (1×10^6 cells/mL, 2×10^7 cells in total) of rat hepatocyte spheroids in a custom serum-free medium and UW solution was observed.²⁹ The conclusion in that paper was based on viability and albumin production. The experiments were performed in serum-free medium supplemented with additives, but ammonia clearance ability after cold storage decreased quickly. This brings up the question: is albumin production a sensitive evaluation indicator? In our preliminary experiments, we found that even though there were no significant differences in albumin production, the ammonia clearance can show great differences. The discrepancy demonstrated that it is more effective to use ammonia clearance rate to evaluate hepatocyte spheroids.

The present study has demonstrated that in the isolated hepatocyte spheroids: (a) the HTK solution supplemented with NAC is superior to HTK solution alone, and similar to HTK supplemented with DEF and DEF + NAC in protecting from injury after cold storage up to 48 hours; (b) HTK solution supplemented with DEF is superior to HTK solution and HTK + DEF after cold storage up to 72 hours. There was an additional protective effect of NAC but with no statistical significance. We also concluded that ammonia clearance rate is a better index to evaluate the status of hepatocyte spheroids. These findings suggest that HTK supplemented with DEF showed more obvious beneficial effects after cold storage of high density of porcine hepatocyte spheroids to tolerate up to 72 hours of cold storage in viability and functionality. The porcine hepatocyte spheroids were above the cutoff criteria for use in a spheroid bioartificial liver. It may fulfill the demand of most of the worldwide transportation. The current study possibly allows for cold storage of hepatocytes derived from genetically engineered pigs such as FAH-deficient pigs in the future.

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Abbreviations:

Arg1 arginase 1

Asl	argininosuccinate lyase
Ass1	argininosuccinate synthase 1
Cps1	carbamoyl phosphate synthase 1
DEF	deferoxamine
FAH	fumarylacetoacetate hydrolase
GSH	glutathione
HTK	histidine-tryptophan-ketoglutarate solution
NAC	<i>N</i> -acetyl-L-cysteine
Nags	<i>N</i> -acetylglutamate synthase
Otc	ornithine transcarbamylase
ROS	reactive oxygen species
SFM	spheroid forming medium
UW	University of Wisconsin solution

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FIGURE 1.

Experimental design. Primary porcine hepatocytes were freshly isolated and rocked continuously at a frequency of 0.12 Hz to induce spheroid formation. After 24 h, the spheroids were collected and underwent continuous culture (control group) and four cold storage conditions (HTK; HTK + DEF; HTK + NAC; and HTK + DEF+NAC). After cold storage (24, 48, and 72 h), the spheroids were placed in 20 mL spheroid forming medium at the density of 2×10^7 in a 5% CO₂, 37°C incubator for 24-h recovery, and the following measurements. Hollow circle: recovery point; solid circle: measurement point. CS, cold storage; DEF, deferoxamine; HTK, histidine-tryptophan-ketoglutarate; NAC, *N*-acetyl-L-cysteine

FIGURE 2.

Characteristics of porcine hepatocyte spheroids. A, Fresh isolated porcine hepatocytes. B, Hepatocyte spheroids after 24-h rocker culture. C, Spheroid number in each spheroid formation. D, Diameter measurement using Multisizer. Scale bar = 50 μm

FIGURE 3. Vital staining and percent viability of hepatocyte spheroids. A, Vital staining by Fluoroquench™ of spheroids was performed to determine the cell viability for each cold storage condition after 24, 48, and 72 h. Hepatocytes were viable (green) or necrotic (red/orange). Scale bar = 50 μm. B, The mean viability of hepatocyte spheroids was calculated from red and green fluorescence. Viability = (mean green fluorescence/[mean red fluorescence + mean green fluorescence]) × 100%. #*P* < 0.05 vs control group

FIGURE 4.

Albumin production and gene expression. A, Expression of albumin gene was examined for each condition by qRT-PCR after 24, 48, and 72 h. Control group 0-h values served as calibrators to determine the relative expression of gene at each time point and group. B, The concentration of porcine albumin was determined by enzyme-linked immunosorbent assay (ELISA) for each condition after 24, 48, and 72 h. # $P < 0.05$ vs control group. * $P < 0.05$ vs HTK group

FIGURE 5.

Ammonia clearance and expression of liver-specific genes. A, Detoxification of ammonia for each cold storage condition after 24, 48, and 72 h. B, Expressions of the six genes of the urea cycle including arginase 1 (Arg1), argininosuccinate synthase 1 (Ass1), argininosuccinate lyase (Asl), carbamoyl phosphate synthase 1 (Cps1), *N*-acetylglutamate synthase (Nags), and ornithine transcarbamylase (Otc) were examined by qRT-PCR for each group after 24, 48, and 72 h. Control group 0-h values served as calibrators to determine the relative expression of gene at each time point and group. C, Expressions of liver-related genes including Cyp1a2, Cyp2e1, and hepatocyte nuclear factor 4 (Hnf4) were examined by qRT-PCR for each group after 24, 48, and 72 h. Control group 0-h values served as calibrators to determine the relative expression of gene at each time point and group. D, The

slope of ammonia clearance in hepatocyte spheroids was calculated. Clearance slope =

$$\frac{C_{\text{Final}} - C_{\text{Initial}}}{t} \#P < 0.05 \text{ vs control group. } *P < 0.05 \text{ vs HTK group}$$

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