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• BASIC RESEARCH •

Cryopreservation and gel collagen culture of porcine hepatocytes

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Abstract

AIM: To study the method of cryopreserving porcine hepatocytes and gel collagen culture measure after its cryopreservation.

METHODS: Hepatocytes, isolated from Chinese experimental suckling mini-pigs by two-step perfusion with collagenase using an extra corporeal perfusion apparatus, were cryopreserved with 50 mL/L to 200 mL/L DMSO in liquid nitrogen for 4 mo, then thawed and seeded in 1 or between 2 layers of gel collagen. The expression of porcine albumin message RNA, cellular morphology and content of aspartate aminotransferase (AST) and urea nitrogen (UN) were examined during culture in gel.

RESULTS: Viability of 150 mL/L DMSO group thawed hepatocytes was (83±4)%, but after purification, its viability was (90±5)%, attachment efficiency was (86±7)%, the viability of thawed hepatocytes was near to fresh cells. When the thawed hepatocytes were cultivated in gel collagen with culture medium adding epidermal growth factor, the hepatocytes grew in various administrative levels in mixed collagen gel, and bunchy in the sandwich configuration cultures. For up to 10 days' culture, the typical cellular morphological characteristics of cultivated hepatocytes could be observed. The leakage of AST was lower during culture in gel than that in common culture. At the same time, the UN synthesized by cells cultivated in mixed gel collagen was higher than that in other groups.

CONCLUSION: Storage in liquid nitrogen can long keep hepatocytes' activities, the concentration of 150 mL/L DMSO is fit for porcine hepatocytes' cryopreservation. Thawed hepatocytes can be cultivated with collagenous matrix, which provides an environment that more closely resembles that *in vivo* and maintain the expression of certain liver-specific function of hepatocytes.

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INTRODUCTION

Bioartificial liver support systems (BALSS), which employ freshly isolated primary hepatocytes, present severe logistic difficulties in the continuous supply of hepatocytes[1-3]. Stored frozen hepatocytes that are thawed as required would solve this problem. Now, storing hepatocytes in liquid nitrogen was an important method to keep its biological abilities, suitable preserving measure can offer high vigorous hepatocytes for several researches. With the further development of related subjects, long-term preservation methods and active architecture of hepatocytes were needed badly to meet the immediate requirement of bioartificial liver and correlated researches[4-7]. Cryopreservation of hepatocytes is essential for the emergent treatment of acute liver failure. Morsiani *et al* reported the clinical use of such thawed cells. These clinical reports certainly reflect the progress made to date in the isolation and handling of hepatocytes, and it is hoped this will lead to a wider use of cellular therapies in liver diseases[8-13].

In order to find a suitable cryopreservation and culture measure, hepatocytes from newly born Chinese experimental mini-pigs were cryopreserved in -196 ℃ for 4 mo, then thawed hepatocytes were cultivated with mixed gel or sandwich gel collagen, and their morphology and biological functions were compared. So this study was to develop a cryopreservation protocol for long-time preparation of porcine hepatocytes, and to determine an optimal purification procedure and culture methods for thawed hepatocytes. At last it could provide important experimental data for the research of bioartificial liver and hepatocytes transplantation.

MATERIALS AND METHODS

Isolation of porcine hepatocytes

Pig hepatocytes were harvested from newly born Chinese experimental mini-pigs (Experimental Animal Center, Third Military Medical University) using the two-step collagenase perfusion method that was modified from the original methods developed by Seglen *et al*[4,20-22]. Briefly, the animals were anesthetized with barbital (30 mg/kg, b.w, intraperitoneally) and their livers were removed. The liver was first perfused *in vitro* via the portal vein with warmed (37 ℃) Ca^{2+} and Mg^{2+} free Hanks balanced salt solution at a flow rate of 20-30 mL/min for 10-15 mL/min, and then perfused with 0.5 g/L collagenase (Sigma, Type IV) in the same solution supplemented with 5 mM $CaCl_2$ and 50 mM HEPES. The reperfusion with collagenase solution lasted 20 min at a rate of 20 mL/min at 37 ℃. After 10 min of incubation (37 ℃) with gentle shaking, the cells suspension was filtered and centrifuged at 50 g, 3 min for 3 times. The viability of the isolated liver cells was determined using standard trypan blue exclusion, ranged from 89% to 98%[14-18].

Cryopreservation of porcine hepatocytes

Isolated hepatocytes were slowly resuspended and gently mixed at 5×10^9cells/L concentration in different cyropreserving solution which consists of 200 mL/L fetal bovine serum (FBS) and 50 mL/L, 100 mL/L, 150 mL/L or 200 mL/L dimethyl sulphoxide (DMSO, Sigma, America), then placed hepatocytes in 1 mL freezing tube (AXYGEN, America), tagged and put into isopropanol. The concrete freezing procedure was: put in room temperature for 15 min; 4 ℃ for 20 min; -20 ℃ for 30 min;

and stayed overnight at -80 ℃, at last, liquid nitrogen for long-term preservation[7,19-23].

Thawing and removal of DMSO

Four months later, the vials were removed from liquid nitrogen and rapidly thawed by immersion in a 37 ℃ water bath. Immediately after thawing, dimethyl sulfoxide was removed by successive dilutions in 50 mL tubes, 20 mL of Leibowitz-15 medium containing 100 mL/L FBS was gently added to 1.0 mL of cell suspension at 3 min of intervals. The cells were washed three times by centrifugation for 3 minutes at 30 *g* and 4 ℃ to remove cryoprotectant. Then hepatocytes pellet was resuspended in L-15 medium with 100 mL/L FBS. Cell viability was estimated by trypan blue dye exclusion. 1×10^6 thawed hepatocytes were centrifuged at 1 000 rpm for 10 min, the supernatants of medium discarded, pellet cells fixed by 30 mL/L glutaral and 10 mL/L OsO_4*etc*, and hepatocytes' ultrastructure was observed through transmission electron microscope[24,25].

Percoll purification of thawed hepatocytes

Working Percoll solution I was prepared by adding 1 part of 10 concentrated D-phosphate-buffered saline into 9 parts of Percoll. Then working Percoll solution II was consisted of 30 mL working Percoll solution I and phosphate-buffered saline. Hepatocytes purification was made by a procedure: to 10 mL of cell suspension, 25 mL of working Percoll solution II were added. The cells pellet was collected by centrifugation at 500 g during 3 min, resuspended, and purified hepatocytes were washed 3 times by centrifugation for 3 min at 30 *g* and 4 ℃.

Common, mixed collagen gel and sandwich configuration culture of thawed hepatocytes

Then hepatocytes were inoculated at 10^6 cell/ bottle in L-15 medium added by 100 mL/L FBS, 10 μl/L glucagons, 200 U/L insulin and 20 ng/L epidermal growth factor (Sigma), as a control group and the medium was changed every day. The morphologies of hepatocytes were observed under inverted microscope, supernatants of culture cells was stored at -20 ℃ for examination. Viability of cells was determined using standard trypan blue exclusion measure.

The collagen solution was prepared just before its use by mixing three parts of collagen type VII (Sigma) in one part of 4×Dulbecco's modified Eagle's medium (Gibco, America) without bicarbonate (pH 7.4), adjusting its pH to 7.20 with 1N NaOH. One million cells were mixed with 2 mL collagen solution and incubated in T-flasks ($25cm^2$) at 37 ℃. When the mixed collagen matrix was fixed in an hour, the gel was washed slowly and medium was added, and hepatocytes were incubated at 37 ℃ with 100 mL/L CO_2 900 mL/L humidity. The methods of culture and observation were the same to the control group[26,27].

When thawed hepatocytes were cultivated in sandwich configuration, the collagen solution was prepared just as before, then T-flasks were coated with it and incubated one hour at 37 ℃. One million cells were cultivated with L-15 medium. Nonattached hepatocytes were washed after one hour; second layer of collagen solution was spread to sandwich the hepatocytes after 24 h. Thirty minutes later, sandwich configuration hepatocytes were formed[28-30], and the methods of culture and observation were just performed as before.

Expression of porcine hepatocytes albumin message RNA

When hepatocytes were cultivated for one week, its total RNA were distillated by Tripure isolated reagent (the gel cultured hepatocytes were digested with 0.5 g/L pancreatin before distillated). The sequences of primers are designed in Table 1. "House-keeping gene" glyceraldehydes-3-phosphate dehydrogenase (G3PDH) gene was kept as inner standard[31].

Table 1 Nucleotide sequences of primers and band sizes

Primers		Sequences (5'-3')	Band sizes
mRNA	sense	CTTATTCCAGGGGTCTGTTTC	324 bp
	antisense	TCGTTTCTCTCAGGCTCTTCT	
G3PDH	sense	CATCATCCCTGCTTCTACCC	160 bp
	antisense	CCTGCTTCACCACTTTCTTG	

RNA was reverse transcribed using a one-step RT-PCR kit (Promga, America). For RT-PCR amplification, the standard RT-PCR program of one cycle of 48 ℃ for 45 min and 94 ℃ for 4 min, 35 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min and one cycle of 72 ℃ for 7 min were applied. The products of RT-PCR were electrophoresised on 15 g/L agarose gel, and figures were gained on Gel-Doc™2000 (BIO-RAD, America). The densities of these straps were scanned, and the odds of every group's band/ corresponding G3PDH strap were seen as the expression of objective gene.

Aspartate aminotransferase (AST) and urea nitrogen (UN)

The level of AST and UN, in culture supernatants of the control group, mixed gel and sandwich configuration, were detected with biological analysis meter (HITACH. Japan)[32].

Data statistics

The data was analysed by the duplication measurement analysis of variance or one-way analysis of variance on SPSS 11.0 software. Results were expressed on mean±standard deviation (mean±SD).

RESULTS

Survival rate and attaching efficiency of hepatocytes

Fresh isolated and cryopreserved/purified hepatocytes were assessed for viability and function by trypan blue dye exclusion. The yield of cells was 1.5 to 5×10^9 cells/liver. Cell viability was (91±4)%, and attachment efficiency of viable hepatocytes after 24 h culture was (89±5)%. After cryopreservtion, the viabilities of 50 mL/L, 100 mL/L, 150 mL/L and 200 mL/L DMSO group of hepatocytes were in Table 2. However, when the 150 mL/L DMSO group thawed hepatocytes were purified by Percoll, its viability was (90±5)%, attachment efficiency was (86±7)%. The viability of thawed hepatocytes were near to fresh cells.

Table 2 Viability and 24 hours attaching efficiency of thawed hepatocytes (%)

Viability / group (mL / L)	50 DMSO	100 DMSO	150 DMSO	200 DMSO
Viability	51±3	71±4[a]	83±4[c]	79±5[c]
Attaching efficiency	32±6	70±5[a]	81±5[c]	77±6[a]

n=5, [a]P<0.05 *vs* 50 mL / L or 100 mL / L DMSO group, [c]P<0.05 *vs* 50 mL / L DMSO group.

In this table, we can see the viability of 100 mL/L DMSO group cells were much better than that of 50 mL/L and 100 mL/L DMSO groups (P<0.05), but there was no significant difference between the group of 150 mL/L DMSO and 200 mL/L DMSO (P>0.05).

Morphology of thawed hepatocytes

The ultrastructure of thawed hepatocytes in 150 mL/L DMSO group were kept well, number of mitochondria was normal, with slight swellen cristae, rough endoplasmic reticulum decreased, smooth endoplasmic reticulum and golgi apparatus increased,

chromatin in cell nucleus distributed normal (Figure 1A).

Thawed hepatocytes of 150 mL/L DMSO group attached to dishes within 4 h (Figure 1B). After 24 hours' culture, cells appeared polygonal, containing granular cytoplasm with one or two centrally located nuclei. Seven days later, they were more flattened and appeared bigger than 24 h cultured hepatocytes. Morphology and ultrastructure of other groups' hepatocytes were abnormal, thus the hepatocytes of 150 mL/L group were provided for gel collagen culture.

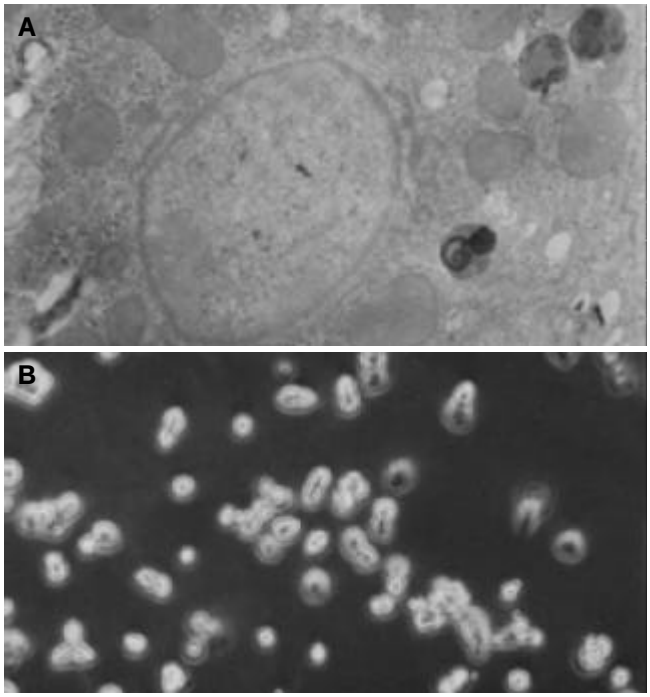

Figure 1 A: The thawed hepatocytes' ultrastructure after 4 mo cryopreservation, (TEM, ×4000), B: The morphology of thawed hepatocytes after 4 h culture (PCM, ×200).

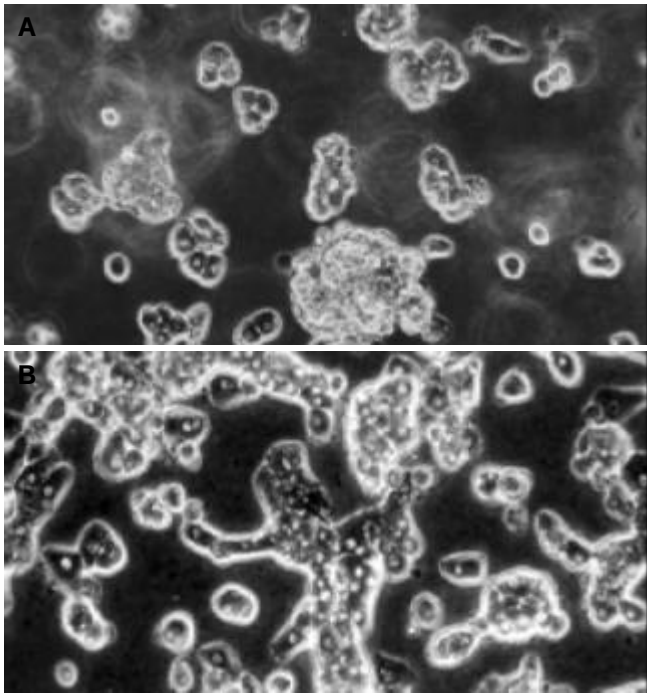

Figure 2 A: Morphology of thawed hepatocytes cultivated in mixed gel for 4 h (PCM, ×200), B: Morphology of hepatocytes cultivated in sandwich configuration for 24 h (PCM, ×200).

Morphology of mixed gelled cultivated pig hepatocytes

The hepatocytes kept their biological characters well after 150 mL/L DMSO cryopreservation. When the thawed hepatocytes were mixed in gel collagen and cultivated with medium adding epidermal growth factor, the hepatocytes grow in various administrative levels in collagen gel. For up to 7 days' culture, the typical cellular morphological characteristics of hepatocytes could be observed (Figure 2A).

Morphology of sandwich culture pig hepatocytes

Hepatocytes, cultivated for four hours in first layer gel, turned from pellet to polygon. When second layer gel collagen was added, hepatocytes cultured in collagen sandwich configuration had a cuboid, compact, bunchy, and well-defined shape with a classical cobblestone appearance. In the seven-days' culture period, the hepatocytes maintained their typical morphological characteristics under phase contract microscope (Figure 2B).

Expression of albumin mRNA on thawed porcine hepatocytes

When the control group mixed gel collagen and sandwich configuration porcine hepatocytes were cultivated for seven days, they all kept expression of albumin message RNA using RT-PCR, but the level of mixed gel collagen group was higher than that of other group ($P<0.05$), (Figure 3 and Table 3).

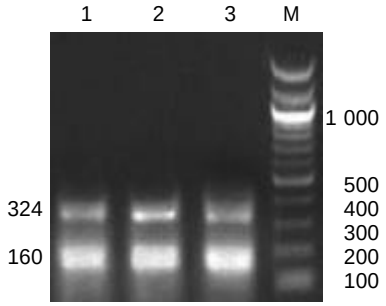

Figure 3 Expression of porcine albumin mRNA on culture hepatocytes after RT-PCR. M: DNA markers; lanes 1, 2 and 3 are control groups, mixed gel collagen and sandwich configuration.

Table 3 Change of albumin mRNA expression in culture pig hepatocytes (mean±SD)

Band density / group	Mixed gel group	Sandwich group	Control group
Mean rate of density	0.44±0.041[a]	0.353±0.021[c]	0.261±0.02

$n=3$, [a]$P<0.05$ *vs* group of control or sandwich; [c]$P<0.05$ *vs* control group.

Release of AST

The level of AST in common culture hepatocytes supernatant was very lower on day 1 to day 3, and ascended to the peak on day 7, which was marked higher than the group of mixed gel collagen or sandwich collagen ($P<0.05$). The contents of AST in the group of gel collagen on culture 24 to 48 h were high, but they dropped at day 3 and kept at a lower level ($P>0.05$), (Figure 4).

UN in culture supernatant

The levels of urea nitrogen synthesized by hepatocytes in three groups were different and changed. The content in common culture group decreased on day 5, and was lower than that of other groups on culture period ($P<0.05$). Mixed gel group's level was higher than that of the sandwich, but they had no marked difference. ($P>0.05$), (Figure 5).

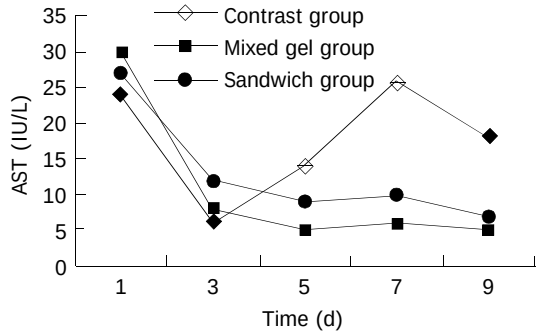

Figure 4 Content of AST on hepatocytes culture medium.

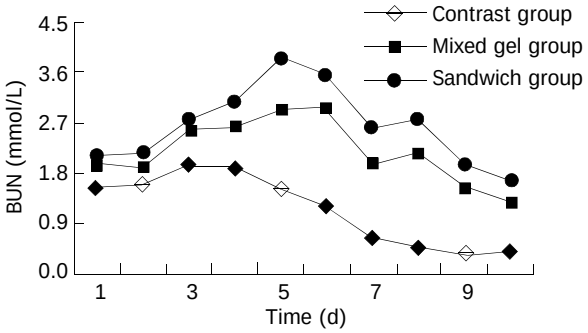

Figure 5 Content of urea nitrogen on hepatocytes culture medium.

DISCUSSION

Hepatocytes cryopreserved in liquid nitrogen were thought as only efficient measure to keep its ability currently[33-36]. Shortage of hepatocytes necessitates the development of improved cryopreservation techniques for long-term storage of hepatocytes to make best use of available hepatocytes. The ultimate goal of any improved cryopreservation protocol is to minimize sudden intracellular formation of ice crystals that could result in ultrastructural damage, and thus maintains cell viability, attachment, and metabolic activity on thawing. Storage time of cryopreserved hepatocytes at temperatures -196 ℃ may play an important role in the quality of thawed cells. Most of the available protocols use dimethyl sulfoxide in the cryopreservation medium[19,37-39].

Successful cryopreservation of porcine hepatocytes would ensure the accessibility of cells for laboratory use, permit the standardisation of experiments, save lives of animals and lead to continuous supply of hepatocytes in BALSS treatment[28,31]. Therefore, we sought the optimal procedure for cryopreservation of porcine hepatocytes for both laboratory and clinical purposes in this study. The protocol for cryopreservation of hepatocytes included the concentration of 150 mL/L DMSO, 200 mL/L fetal bovine serum and rapid thawing followed by slow dilution of DMSO to avoid osmotic shock. After 4 mo cryopreservation, the thawed cells were shown to have a reasonable level of cell viability, compared to freshly isolated hepatocytes. So the storage condition was proved to be suitable for long-time cryopreservation of pig hepatocytes. Our purification method using D-PBS and Percoll achieved a high cell recovery rate with satisfactory viability and function at high cell concentrations (5×10^9cell/L), facilitating purification of hepatocytes in large quantities. We conclude that frozen/thawed/purified cells can possess satisfactory viability and function. Large-scale cryopreservation of porcine hepatocytes may provide an efficient alternative to freshly isolated porcine hepatocytes[19, 40-42].

Proper cryopreservation measure can maintain porcine hepatocytes' abilities for a long time. However, if the thawed hepatocytes were cultivated in unfit conditions, it would not meet the need of application in clinic and basic research. Therefore, the culture pattern of thawed hepatocytes also appeared very important. Traditional cell culture measure, which was common monolayer attaching incubation, was hard to simulate the circulation that hepatocytes grew *in vivo*, and were not suitable for BLASS[43]. Collagen was a kind of matrix in common use to fix hepatocytes. Liver cells *in vivo* assume three-dimension configuration in a liver lobule, and there are complicated contacts among hepatocytes, liver non-parenchyma cells and cell matrix. Matrix is an important part of affecting hepatocytes function. Many researches showed that collagenous matrix could provides an environment that more closely resembles that *in vivo* and allows to maintain the expression of certain liver-specific function of hepatocytes[44-47]. In this study, the hepatocytes keep their biological characters well after storage in liquid nitrogen for 4 mo. When the thawed hepatocytes were cultivated in gelled collagen with culture medium added by epidermal growth factor, the cells grow in various administrative levels in mixed collagen gel, and bunchy in the collagen sandwich cultures. For up to 10 days' cultures, the typical cellular polygonal morphological characteristics of culture hepatocytes could be observed, and the bound between hepatocytes appear brightly and clearly; the leakage of aspartate aminotransferase was less and the level of urea nitrogen synthesized by hepatocytes was higher. Through sandwich culture can hold hepatocytes synthesizing and metabolizing abilities, the cultivated hepatocytes was also at monolayer attaching state, and do not have normal three-dimension construction *in vivo*, so it needs to be further ameliorated[26,29,43].

In conclusion, through the study of many important factors in hypothermic preservation, we can optimize the hypothermic preservation protocols or cultivated conditions, and established a hepatocytes storage room. That will be helpful for the development of BAL and hepatocytes transplantation[9,40,48].

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