ARTICLE IN PRESS

Cryobiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

The effects of over expressing aquaporins on the cryopreservation of hepatocytes

Balasubramanian K. Kumar [a], Robin N. Coger [b], Laura W. Schrum [c,d], Charles Y. Lee [a,d,*]

[a] Department of Mechanical Engineering and Engineering Science, University of North Carolina, Charlotte, NC, United States
[b] College of Engineering, North Carolina A&T State University, Greensboro, NC, United States
[c] Department of Internal Medicine, Carolinas Medical Center, Charlotte, NC, United States
[d] Center for Biomedical Engineering and Science, University of North Carolina, Charlotte, NC, United States

ARTICLE INFO

Article history:
Received 25 June 2014
Revised 9 June 2015
Accepted 22 July 2015
Available online xxxx

Keywords:
Liver
Hepatocytes
Cryopreservation
Aquaporins
Rat

ABSTRACT

During cryopreservation, aquaporins are critical in regulating water transport across cellular membranes and preventing osmotic damages. Hepatocytes express aquaporin (AQP) 0, 8, 9, 11, and 12; this study investigates whether increasing the localization of AQP8 on the cellular membrane would improve cell viability by increasing water transport during cryopreservation. Primary rat hepatocytes were cultured and treated with dibutyryl cAMP (Bt_2cAMP) or glucagon to increase the expression of AQP8 at the cellular membrane via translocation. This phenomenon is verified through two experiments – confocal immunofluorescence microscopy and cell shrinkage analysis. The immunofluorescence results showed increase in AQP8 on the cellular membrane of treated cells, and cell shrinkage analysis showed an increase in water transport of treated cells compared to controls. Primary rat hepatocytes were treated with Bt_2cAMP or glucagon and cryopreserved using standard protocols in a controlled rate freezer. This resulted in a significant increase in the cell viability on warming. These results indicate that Bt_2cAMP or glucagon treated hepatocytes had increased expression of aquaporin in the cellular membrane, increased water transport during cryopreservation, and increased post-thaw viability.

© 2015 Published by Elsevier Inc.

1. Introduction

Water transport has been shown to be one of the most important factors in cell survival after cryopreservation. [12,15,23]. The osmotic gradient created during the freezing process and the limitation of water transport results in increased incidence of intracellular ice formation leading to higher incidence of cell death [13,26]. The presence of water channels, known as aquaporins (AQPs), a family of integral membrane proteins, facilitate water movement due to osmotic gradients across the cellular membrane [24]. Of the 13 isoforms discovered so far, five were identified to be expressed in hepatocytes: AQP0, AQP8, AQP9, AQP11 and AQP12 [1,4,5,11,16,22]. Among these, AQP8 is localized in the plasma membrane [20], intracellular vesicles and the mitochondria. Prior experimental evidence shows that AQP8 has a tendency to translocate to the cellular membrane on the influence of choleretic stimulus [6,7]. Therefore, we hypothesize that increasing presence of aquaporin on the cellular membrane by translocation of AQP8 from the intracellular vesicles can help increase the water transport and improve cryopreservation of cultured hepatocytes.

Thus, in this study, the increase of aquaporin by treatment with dibutyryl cAMP (Bt_2cAMP) or glucagon and its effect on the post-thaw viability of rat primary hepatocytes are evaluated. The translocation of AQP8 via the stimuli is evaluated by immunofluorescence staining and cell shrinkage analysis. Viability of the cells is assessed by Live-Dead staining.

2. Materials and methods

2.1. Hepatocyte isolation

Procedures adhered to NIH's Institutional Animal Use and Care Guidelines. Sprague–Dawley male rats weighing 150–280 g were fasted 24 h prior to isolation, and hepatocytes were isolated by collagenase perfusion method [18]. In brief, the rat liver was perfused with collagenase solution for approximately 10 min. The hepatocytes from the digested liver were isolated by mechanical disruption and filtering through a nylon mesh (105 µm). The hepatocytes were then separated from the nonparenchymal cell

* Corresponding author at: Department of Mechanical Engineering and Engineering Science, Center for Biomedical Engineering and Science, UNC Charlotte, 9201 University CIty Blvd, Charlotte, NC 28223, United States.

E-mail address: cyclee@uncc.edu (C.Y. Lee).

http://dx.doi.org/10.1016/j.cryobiol.2015.07.010
0011-2240/© 2015 Published by Elsevier Inc.

fractions by centrifugation (Thermo IEC CEntra-CL3R, Thermo Scientific, MA) at 50*g* for 3 min. The viability of the centrifuged hepatocytes was evaluated immediately using trypan blue exclusion assay (Sigma–Aldrich, St. Louis, MO). If the resulting viability was lower than 90%, percoll (GE healthcare, Waukesha, WI) centrifugation was performed to achieve a minimum of 90% viability for the cell culture. Then the hepatocytes were re-suspended in culture media containing DMEM (Invitrogen, Gaithersburg, MD), sodium bicarbonate (3.7 g/L), insulin (500 U/L), epidermal growth factor (20 μg/L), hydrocortisone (7.5 mg/L), 1% (v/v) of antibiotic/antimycotic solution (JR Scientific, Woodland, CA) and 10% (v/v) fetal bovine serum (HyClone, Thermo Scientific, Waltham, MA).

2.2. Culture of hepatocytes

Collagen type I gel based single gel culture of hepatocytes in 35 mm diameter tissue culture plates were used for most of the experiments. The collagen gel was first prepared by adding 8 parts of 1.1 mg/mL PureCol collagen (Advanced BioMatrix, San Diego, CA) to 1 part 10× DMEM solution. The pH was adjusted to 7.4 with 0.1 N HCl and/or 0.1 N NaOH. Half a milliliter of the prepared collagen was then coated on each 35 mm diameter tissue culture plate and incubated for one hour at 37 °C, 5% CO_2 for gelation. Cells (2×10^6) were seeded in each tissue culture plate, and 1 mL of DMEM media was added and incubated at 37 °C, 5% CO_2. The media was changed after 3 h to remove the unattached cells and incubated for 24 h.

2.3. Treatment of the hepatocytes

After 24 h of incubation, hepatocytes were treated with (a) dibutyryl cAMP (Bt_2cAMP) (100 μM) or (b) glucagon (1 μM) (both Sigma–Aldrich, St. Louis, MO) and incubated for 12 h. For the controls, 1 mL of normal DMEM media was added to each culture plate and incubated for the same period as the treated ones. After 12 h of incubation, some cells were treated with 0.1 mM $HgCl_2$, a water channel inhibitor, for 5 min. In addition, a group of control cells that did not experience freezing was also treated with $HgCl_2$ for 24 h to assess the toxicity of $HgCl_2$ during the testing period.

2.4. Confocal immunofluorescence

For confocal immunofluorescence experiments, collagen coated chamber slides were used. Hepatocytes (5×10^5) were plated on the collagen-coated chamber slides and incubated at 37 °C for 4 h. The cells were then treated with (a) Bt_2cAMP, (b) glucagon or (c) normal DMEM media (control) and incubated for 12 h. After the 12 h, the cells were fixed with 2% formaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 2 min. The cells were then treated with a blocking solution containing 3% BSA at room temperature and incubated overnight at 4 °C with goat affinity-purified AQP8 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) of dilution 1:50 in PBS. Then, the chamber slides were rinsed with PBS solution and treated with Alexa Fluor 488 – conjugated donkey anti-goat HRP secondary antibody (Invitrogen, CA) for 1 h. The dilution of the secondary antibody used was 1:400 in PBS. Then the cells were treated with 1 μg/mL concentration of Hoechst 33,342 (Molecular Probes, Eugene, OR) and mounted with Pro-Long (Molecular Probes, Eugene, OR). Fluorescence localization of AQP8 was then detected by immersion oil confocal microscopy (Olympus Fluoview FV500) with 100× magnification lens.

2.5. Cell shrinkage analysis

Cell shrinkage analysis was performed on treated and control samples prepared on tissue culture plates as described above. One culture plate at a time was transferred to an Olympus IX70 microscope (Olympus America Inc., PA) mounted with a computer interfaced camera (Hamamatsu Corporation, Bridgewater, NJ). Media from each culture plate was aspirated and 1 mL of 5 M NaCl solution was added. Response of cells to the hypertonic NaCl solution was captured at 40× magnification at one minute intervals for 20 min. Images were processed using software MetaMorph Imaging System (Molecular Devices, Sunnyvale, CA). Using MetaMorp, the variations of the cross-sectional area of the cells at various sites were measured over time to analyze the shrinkage behavior of the cells in the hypertonic solution.

2.6. Cryopreservation of treated and control samples

After treatment of cell cultures for 12 h, samples were removed from the incubator and placed on ice to reduce the cell temperature to 4 °C to minimize the toxicity of the cryoprotecting agent (CPA). Two different CPA solutions, 20% dimethyl sulfoxide in DMEM media and 20% glycerol in DMEM media were used. The CPA solution (1 mL) was added to the samples and incubated at 4 °C for 10 min to reach equilibrium. Samples were transferred to the CryoMed Control Rate freezer (Thermo Forma, Waltham, MA).

The controlled cooling process was initiated at 4 °C and a cooling rate of 1 °C/min was maintained until −16 °C followed by a cooling rate of 2 °C/min until −36 °C/min, and thereafter a cooling rate of 10 °C/min until a temperature of −80 °C was achieved. The samples were further maintained at −80 °C for 5 min to ensure equilibrium. At the end of the freezing process, the samples were transferred immediately to a −80 °C Revco freezer (Kendro Laboratory, Ashville, NC) and stored for a week.

2.7. Evaluation of post-thaw cell viability

The cryopreserved samples from the −80 °C freeze were transferred to a sterile glass box and plated in a water bath maintained at 37 °C until the media in the frozen samples completely thawed. At this juncture, the samples were between 5 and 10 °C. Immediately, the CPA containing media in the samples was aspirated to prevent any toxicity to the cells. DMEM (1 mL) was added to the samples and incubated at 37 °C for 10 min. After 10 min of incubation, the media in the samples were refreshed in order to remove any remaining traces of CPA. These samples were then placed in the incubator at 37 °C, 5% CO_2 for 24 h and allowed to recuperate from the freeze–thaw process.

After 24 h of recuperation time, cell viability of the samples was determined by using nuclei fluorescence dyes. The samples were washed with 1XPBS solution and incubated with Hoechst Dye (1 μg/mL) and ethidium homodimer (2 μM) (Molecular Probes, Eugene, OR) in PBS for 30 min. The viability solution was aspirated, and samples were fixed by adding 10% formalin (1 mL) (VWR, West Chester, PA) and incubating for 20 min. Subsequently, cell viability was examined with a confocal microscope with DAPI (excitation 358 nm; emission 461 nm) and Texas red (excitation 596 nm; emission 620 nm) filters. The fluorescent images obtained were then analyzed using MetaMorp Imaging System.

2.8. Statistical analysis

One-way Analysis of Variance (ANOVA) was performed to determine the significant differences for all the data analyses. All the analyses were considered a two tailed test with type I error, α as 5%. Most of the experiments were performed for doublet samples (in some case triplicates) and each experiment was repeated for a minimum of three rats.

Please cite this article in press as: B.K. Kumar et al., The effects of over expressing aquaporins on the cryopreservation of hepatocytes, Cryobiology (2015), http://dx.doi.org/10.1016/j.cryobiol.2015.07.010

Download English Version:

https://daneshyari.com/en/article/10927860

Download Persian Version:

https://daneshyari.com/article/10927860

Daneshyari.com