Effect of dithiotheritol on viability of cryopreserved rat hepatocytes

*[1]Jamshidzadeh A., [2]Hosseinaghdaie M., [1]Niknahad H., [1]Nematizadeh M.

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, and Pharmaceutical Sciences, Research Center, [2]Transplant research center, Shiraz University of Medical Sciences, Shiraz, Iran, 71345-1662.

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ABSTRACT

Background: Prolonged storage of cryopreserved isolated hepatocytes is now possible and such cells have been used with success for drug metabolism and toxicity studies. Quality of the cells before cryopreservation is important for viability and function after freezing.

Methods: In this study, fresh rat hepatocytes were incubated with the thiol-containing compounds N-acetylcysteine (NAC), dithiotheritol (DTT) and fructose as ATP supplier, at incubation times 1 and 3 hrs. The preincubated hepatocytes cryopreserved for 24 hour, and 1 and 3 months. Hepatocytes, viability were determined immediately postthaw by Trypan Blue exclusion.

Results: Fructose preincubation improved the viability of hepatocytes at a concentration of 300 mM. Preincubation with DTT (50, 100 and 200 μM) prior to cryopreservation had beneficial effects on viability of hepatocytes. The postthaw viability of hepatocytes preincubated with NAC was uniformly poor.

Conclusion: Preincubation with DTT, improved GSH levels before freezing which could be responsible for the reduction in membrane damage during cryopreservation. It may be concluded that the intracellular GSH level is an important determinant in the deleterious effects of cryopreservation procedure.

Keywords: Cryopreservation, Dithiotheritol, Hepatocytes.

INTRODUCTION

The use of primary hepatocytes is now well established for studies of both drug metabolism and enzyme induction. Cryopreservation of primary hepatocytes decreases the need for fresh liver tissue. This is especially important for research with human hepatocytes because availability of human liver tissue is limited. However, their clinical uses, require better metabolic characterization and an optimal cryopreservation procedure in order to improve cell viability and metabolic activity after thawing (1).

There are limited data on preincubation of hepatocytes prior to cryopreservation. Various cryopreservation protocols, mostly for rat hepatocytes have been proposed, over the last 25 years. Although progress has been reported for cultured hepatocytes (2, 3) attempts to cryopreserve isolated primary hepatocytes have resulted in limited success. Reports detailing cell viability have demonstrated high viability, from 30% to 90% immediately after thawing (4-8) and reveal a continuous decline in cell number within a few hrs (9). Additionally, there are several reports that despite high viability, few hepatocytes were able to attach to culture surfaces and survive for extended periods of time (8-10).

Several possible mechanisms for cell death during cryopreservation have been proposed of which the most important mechanisms appear to be oxidative stress and apoptosis (11).

Natural antioxidants exert a protective effect on the plasma membrane in cryopreserved hepatocytes and preserve both metabolic activity and cellular viability. Preincubation of rat and human hepatocytes with cytoprotective compounds prior to cryopreservation have been found beneficial for both hepatocytes (12). It has been shown that a relative to simple step of preincubation prior to cryopreservation of rat and human hepatocytes increases the viability and function of cells upon thawing.

Dithiothreitol (DTT), owing to its antioxidant properties, is widely used in cell biology, biochemistry, and biomedical investigations. This thiol prevents the cytotoxicity of hydrogen peroxide by accelerating its decomposition in culture medium (13).

Based upon previous findings on cryoprotectants, it was hypothesized that the utilization of DTT would improve overall hepatocyte viability through modulation of the cellular biochemical response to the cryopreservation process.

Correspondence: ajamshid@sums.ac.ir

MATERIALS AND METHODS

Dithiothreitol was purchased form Aldrich Chemical Company, Collagenase from Clostridium Histolyticum from Worthington Company (Sidney, Australia), HEPES and BSA from Boehringer-Mannheim (Montreal, Canada), and EGTA, Fructose, NAC and DMSO from Sigma Chemical Company (Cambridge, UK) were used in this study. Other chemicals were of highest grade available.

Hepatocyte Isolation

Male Sprague-Dawley rats (200-250 g) were obtained from the Laboratory Animals Research Center of Shiraz University of Medical Sciences. All animals received human care and the experimental protocol was approved by the Committee of Laboratory Animals according to university guidelines.

Hepatocytes were isolated from rats by a two-step collagenase perfusion technique as described previously (14). Hepatocytes (1×10^6 cells/ml) were suspended in Krebs-Henseleit buffer (pH:7.4), containing 12.5 mM HEPES and kept in rotating, round bottomed 50 ml flasks at 37 °C under a continuous flow of 95% O2 and 5% CO2. The cells were allowed to equilibrate with incubation conditions for 10 min before addition of compounds to the incubation mixture. Hepatocytes with a viability of more than 75% as measured by Trypan Blue Exclusion were used.

Preincubation, Cryopreservation and Thawing

Following isolation, hepatocytes were incubated with fructose (100, 200 and 300 mM), NAC (10, 20 and 40 mM) and DTT (50, 100 and 200 μM) at 7°C and 37°C for 1 and 3 hrs. Two hundred microliters of treated sample were removed at the end of incubation times for trypan blue exclusion study. Then, cells were centrifuged at 50 × g and resuspended in culture media (control), ice-cold HTS (5 mM HEPES/4.5 mM Tris pH 7.4) with 10% DMSO (HTS-cryo; trade name: CryoStor CS10) or ice-cold culture media with 10% DMSO (Media-cryo), at a cell concentration of 2×10^6 cells/mL. Cell suspensions (1.0 mL) were transferred to cooled cryogenic vials and incubated on ice for 10 min, then placed into a controlled rate cooler (Kryo 10; Planer, Middlesex, UK) and frozen under the following protocol. From 4°C, samples were cooled at -1°C/min to -6°C and held for 10 minutes to allow for sample equilibration. Extracellular ice formation was then initiated by application of cold forceps to the exterior of the cryovial. After another 10 min hold, samples were cooled at -1°C/min to −80°C, and then transferred to liquid nitrogen for three times storage. Cryopreserved cells were removed from liquid nitrogen (after 24 hrs, and 1 and 3 months), and immediately immersed in a water bath that had been preheated to 37°C. The vials were shaken gently until the contents were completely free of ice crystals (approximately 90-120 sec) and were then emptied into the prewarmed hepatocyte suspension buffer. The cells were centrifuged at 40*g* for 5 min at 19°C, the supernatant was removed by aspiration, and the resultant pellet was suspended in Williams' medium E (WEM) at a density of 10^6 cells/ml (15). The hepatocytes were placed in plate (96-well) at 37°C, 5% CO2. After 4 hrs, hepatocyte viability was determined by Trypan Blue exclusion. For this analysis, 50 μL of cell suspension was added to 50 μL of Trypan Blue. The numbers of dead and live cells were scored under light microscope.

Statistical analyses

The results are shown as mean ± standard deviation (SD) of at least three different experiments with different batches of hepatocytes. The differences between groups were determined by t-test and one way ANOVA and a $p < 0.05$ was considered significant.

RESULTS

Hepatocytes were isolated from different rat tissue samples yielding cells with a mean fresh viability of 75± 9%. The viabilities of the control cryopreserved hepatocytes after thawing were 56±7, 52±8 and 44±4 %, for 24 hrs, and 1 and 3 months storage in liquid nitrogen respectively. To determine the influence of preincubation temperature and time on the postthaw viability, hepatocytes were incubated at 7°C and 37°C for 1 and 3 hrs before cryopreservation in all groups. For preincubation temperatures, there was no significant difference between 7°C and 37°C (Figure 1). For preincubation times, the highest postthaw viability was observed for 3 hrs of incubation. The postthaw viability for incubated with DTT (100 and 200 μM), NAC (40 mM) and fructose (300 mM) for 3hrs prior to cryopreservation was statistically ($p < 0.005$) greater than that observed for 1hrs incubation time (Figure 2).

The next phase of the investigation involved determining the influence of duration of storage in liquid nitrogen. Preincubated hepatocytes at 37°C for 3 hrs in different groups preserved in liquid nitrogen for 24 hrs, and 1 and 3 months. Cryopreserved samples showed no differences in postthaw viability in different periods of storage in liquid nitrogen (Table 1).

Results show that hepatocytes preincubated with fructose (300 mM) at 37°C had significantly higher viability than control hepatocytes after thawing (Table 1). Also hepatocytes preincubated at 37°C with DTT had significantly higher viability in comparison to control hepatocytes after thawing. There was no significant concentration-dependent increase in the viability for DTT pretreated cells. NAC preincubation at 37°C had no significant

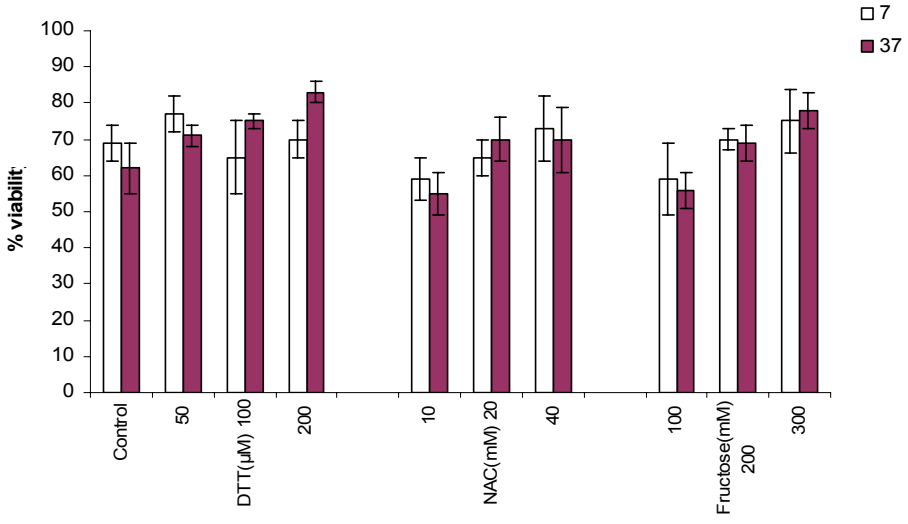

Figure 1. Isolated rat hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH=7.4 under a flow of 95% O_2 and 5% CO_2. The cells were preincubated with DTT, NAC and fructose at 7°C and 37°C for 3 hrs before the cryopreservation. After 24 hrs of cryopreservation, cell viability was assessed by trypan blue exclusion test. Results are mean ± S.D. of at least 3 different experiments.

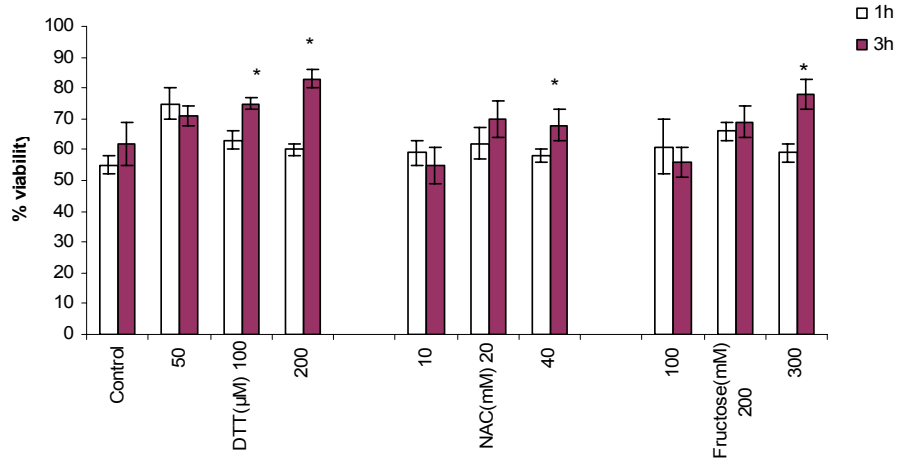

Figure 2. Isolated rat hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH=7.4 under a flow of 95% O_2 and 5% CO_2. The cells were preincubated with DTT, NAC and fructose at 37°C for 1 and 3 hrs before the cryopreservation. After 24 hrs, of cryopreservation, cell viability was assessed by trypan blue exclusion test. Results are mean ±S.D. of at least 3 different experiments.
*: Significantly different between groups of 1 and 3h ($p<0.05$).

effects on the viability in rat hepatocytes after cryopreservation (Table 1).

DISCUSSION

Cryopreserved hepatocytes are a ready source for metabolic and synthetic functions of hepatocytes transplantation and bioartificial livers. Hepatocytes are extremely sensitive to damage during freezing and thawing, even after addition of classical cryoprotectants and as a result improved cryopreservation techniques are required to reduce cell injury and functional impairment of hepatocytes. If the loss of some viable cells during the freeze–thaw process cannot be avoided, conditions of incubation and culture of cryopreserved liver cells will be critical for prolonged maintenance of functional activities (16, 17).

Survival and function of cryopreserved hepatocytes can be improved when these cells have a high energy pool, are cryopreserved after immobilization in a gel, separated from dead cells on a Percoll gradient or placed in more favorable culture conditions (e.g. in coculture with liver non parenchymal cells) (15). This study illustrates the role of DTT and fructose on the ability of hepatocytes to tolerate and survive the stresses of freezing and thawing. As indicated in results, preincubation of the freshly isolated rat hepatocytes with fructose (300 mM), resulted in a statistically significant effects on postthaw viability. Terry et al. have shown that preincubation of both

Table 1. Preincubation of N-acetylcysteine, dithiotheritol and fructose prior to cryopreservation on viability of cryopreserved rat hepatocytes.

		% viability Time of cryopreservation		
		24 h	1 m	3 m
Control		62±7	63±6	57±3
DTT (μM)	50	71±3*	89±5*	76±4*
	100	75±4	81±4*	80±3*
	200	79±3	83±3*	75±7*
NAC (mM)	10	55±6	62±2	60±5
	20	70±6	71±5	62±9
	40	70±5	79±7	60±7
Fructose (mM)	100	56±5	59±4	55±5
	200	69±3	63±8	62±4
	300	78±5	81±3*	66±5

Isolated rat hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH=7.4 under a flow of 95% O_2 and 5% CO_2. The cells were preincubated with DTT, NAC and fructose at 37°C for 3 hrs before the cryopreservation. After 24 hrs, and 1 and 3 months of cryopreservation, hepatocytes were thawed and viability was determined postthaw by Trypan Blue exclusion. Results are mean ± S.D. of at least 3 different experiments.

*: Significantly different from control group ($p < 0.05$).

rat and human hepatocytes with glucose, fructose or α-lipoic acid at 4°C prior to cryopreservation had beneficial effects on the postthaw viability and function of hepatocytes (12). Fructose can protect rat hepatocytes which are cultured in hypoxic conditions against apoptosis by forming additional nicotinamide adenine dinucleotide phosphate (NADPH) for regeneration of reduced glutathione (GSH) via stimulation of the pentose phosphate pathway and thereby reducing generation of reactive oxygen species (18). It has been suggested that adding substrates such as fructose during isolation of hepatocytes may improve the energy recovery of hepatocytes after the stress of ischemia reperfusion and cell isolation (19).

As it is well known, GSH is a crucial component of cellular antioxidant defenses and plays a major role in protecting cells against oxidative stress (20, 21), Therefore, the ability of cells to maintain GSH equilibrium is essential for their functions and survival. Recently, it has been demonstrated that intracellular GSH was depleted before the onset of cell death induced by various insults, and artificially depleting intracellular GSH renders cells more sensitive to toxic chemicals (22, 23). Furthermore, it has been reported that cryopreservation decreased intracellular reduced glutathione up to 50% and inclusion of ascorbic acid and α-tocopherol in the freezing medium improved maintenance of reduced glutathione content post–cryopreserved hepatocytes (24). Results of this investigate indicated that after 3 hrs incubation with 100 and 200 μM of DTT, viability of cells after cryopreservation were significantly increased compared to the control group. Although, NAC increased the viability of the cells, this increase was not statistically significant compared to the control group which is consistent with findings of other study (12). Results of this study suggest that a decrease in reduced glutathione or oxidation of the thiol groups of cellular proteins are mechanisms involved in cellular death during cryopreservation procedure. DTT directly reduced the thiol groups while NAC has to be converted to glutathione after an enzymatic reaction which requires longer time (25). The more significant effect of DTT on viability of cryopreserved hepatocytes compared to NAC is due to the fact that this compound can rapidly reduces both GSSG and pr-SS-pr (protein disulfide bonds), while NAC needs more time to reduce the oxidized thiol groups in the cells. It may be concluded that depletion of protein thiols is one of the possible mechanisms of deleterious effects of cryopreservation procedure.

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