Reduced glutathione levels and expression of the enzymes of glutathione synthesis in cryopreserved hepatocyte monolayer cultures

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Abstract

Cryopreservation of monolayers of hepatocytes in a freezing medium containing 10% (v/v) dimethylsulfoxide, 90% (v/v) foetal calf serum retains cell morphology and viability, but cells lose up to 50% of their intracellular reduced glutathione. This is accompanied by a small increase in glutamate cysteine ligase expression in cryopreserved cultures, but glutathione synthetase expression is undetectable post-cryopreservation. Inclusion of ascorbic acid and α-tocopherol in the freezing medium improves maintenance of reduced glutathione content post-cryopreservation at 84% of the levels in non-cryopreserved monolayer cultures, but does not restore glutathione synthetase expression. The inability to synthesise reduced glutathione will mean that cryopreserved hepatocyte monolayers are more susceptible to toxic insults.

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1. Introduction

Cryopreservation is potentially a useful means of storing hepatocytes for use by scientists in the pharmaceutical industry in in vitro metabolism studies of new candidate drugs. We have previously demonstrated that rat hepatocytes cryopreserved as monolayers retain UDP-glucuronosyltransferase activity and testosterone hydroxylation (a measure of cytochrome P450 activity) (Stevenson et al., 2004). In this protocol, developed by Watts and Grant (1996) and McKay et al. (2001), primary rat hepatocytes are cultured on collagen coated dishes before being cryopreserved. This circumvents the problems of low post-thaw recovery and attachment obtained with hepatocytes frozen in suspension (Diener et al., 1993; Innes et al., 1988). Moreover, in monolayers the hepatocytes are all in close contact with the surrounding media, facilitating nutrient and substrate penetration during metabolism studies. This is a distinct advantage over other hepatocyte model systems such as spheroids, collagen sandwiches and liver slices.

To allow confidence in the data generated by in vitro metabolism studies it is important that the cells used are competent both in generating and detoxifying reactive intermediates. The activity of cytochrome P450 has been demonstrated in the monolayers post-cryopreservation (Stevenson et al., 2004), confirming that generation of reactive intermediates by this pathway can take place effectively. One of the major means of detoxification of reactive species is by conjugation with reduced glutathione (GSH). The intracellular GSH level is an important determinant in the toxic response to many xenobiotics (Reed, 1990), and it is therefore essential that the GSH content of cryopreserved hepatocytes is maintained at normal levels. In this paper, the GSH levels of cryopreserved monolayers of hepatocytes,
the expression of glutamate cysteine ligase (GCL) and glutathione synthetase (GS), and the GSH conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) have been investigated.

2. Materials and methods

2.1. Isolation and culture of primary hepatocytes

All chemicals are from Sigma Chemical Co., Poole, Dorset, UK, unless otherwise stated. Hepatocytes were isolated from male Sprague Dawley rats (180–260 g), which had ad libitum access to food and water, by collagenase (from Gibco BRL Life Technologies, Paisley, Scotland) perfusion (Moldeus et al., 1978). Viability of the hepatocyte suspensions was typically 85–95%, as determined by Trypan Blue exclusion. 50 mm diameter Falcon tissue culture dishes coated with type 1 collagen (0.0435 mg/cm²) isolated hepatocytes colonies attached to the culture surface was quantified using an image analysis programme Scion Image. This information together with the total protein content data indicates the attachment and viability of the cells in both non-cryopreserved and cryopreserved cultures. GSH levels were measured by the method of Hissin and Hilf (1976) and total protein by Lowry et al. (1951). Expression of GS and GCL was measured by immunoblotting using 10 μg of cellular protein. The optical density was quantified using an image analysis programme Scion Image. Antibodies to GS and GCL were supplied by Dr. Lesley McLellan, University of Dundee. GSH conjugation of 50 μM CDNB in 2 ml Krebs–Hepes buffer, pH 7.4, was measured in

Cultures were cryopreserved at 24 h post-isolation for 24 h and compared with their respective non-cryopreserved controls. Assuming biological time stops during cryopreservation, 24 h, 48 h, and 72 h post-thaw cultures (which had been pre-cultured for 24 h prior to cryopreservation) were compared with 48 h, 72 h, and 96 h non-cryopreserved controls, respectively. GSH was measured at 96 h in non-cryopreserved cultures and compared with 72 h post-thaw cultures using the modifications to the freezing medium and protocol described above. These results are expressed on the graphs as % control GSH values. The expression of GCL and GS was measured in non-cryopreserved cultures for up to 96 h, and in cryopreserved cultures for 72 h post-thaw.

2.4. Analytical methods

Cultures were cryopreserved at 24 h post-isolation for 24 h and immediately placed in a polystyrene box with a lid, covered by a thin media were then removed completely, leaving the cultures to 40 min, at 10 min intervals were tested. Cryopreservation medium prior to freezing was investigated. Times from 10 increasing the equilibration time with the cryoprotectant W Watts and Grant, 1996) with minor alterations. Cultures by a previously described protocol (McKay et al., 2001; Borel Rinkes et al., 1992; Li et al., 1999). The effect of addition of the following antioxidants to the freezing medium was investigated: 1 μM α-tocopherol and 1 mM ascorbic acid; 1 mM deferoxamine; 200 U/ml superoxide dismutase and 450 U/ml catalase. The effect of all these antioxidants added together was also investigated. The concentrations of antioxidants used were chosen with reference to previous studies that had proven their antioxidant/cytoprotective action during hypothermic storage or hepatocyte culture (McAnulty and Huang, 1997; Whiteley et al., 1992; Halpner et al., 1998).

2.3. Comparision of cryopreserved and non-cryopreserved hepatocytes

Cultures were stained with 0.1% w/v ethidium bromide and 25 μM carboxyfluorescein diacetate (CFDA) as described previously (Stevenson et al., 2004) and viewed using a Leica confocal laser scanning microscope (CLSM) with a 25× water immersion lens (NA = 0.75). Ethidium bromide staining was viewed at 488 nm emission wavelength using a barrier filter >590 nm, and CFDA at 536 ± 16 nm emission wavelength with a 536/16 nm narrowband filter. Using the CLSM digital images the area of viable hepatocyte colonies attached to the culture surface was quantified by Scion image (freeware package available at <www.scioncorp.com>). This information together with the total protein content data indicates the attachment and viability of the cells in both non-cryopreserved and cryopreserved cultures. GSH levels were measured by the method of Hissin and Hilf (1976) and total protein by Lowry et al. (1951). Expression of GS and GCL was measured by immunoblotting using 10 μg of cellular protein. The optical density was quantified using an image analysis programme Scion Image. Antibodies to GS and GCL were supplied by Dr. Lesley McLellan, University of Dundee. GSH conjugation of 50 μM CDNB in 2 ml Krebs–Hepes buffer, pH 7.4, was measured in
monolayer cultures for up to 30 min, and the formation of the conjugate measured spectrophotometrically at 340 nm. Formation was linear for at least 15 min, and was quantified using the molar extinction coefficient of GSH-CDNB which is $9.8 \text{mM}^{-1}\text{cm}^{-1}$.

3. Results

Fig. 1 shows the morphology of hepatocytes before and after cryopreservation. The cells were stained with the vital stain CFDA, and the images show generation and retention of brightly fluorescent carboxyfluorescein in viable intact cells. Cells show typical hepatocyte morphology. The pictures were taken at 96 h in non-cryopreserved cultures and 72 h post-cryopreservation, and demonstrate retention of viability in the post-thaw cells. The area of the viable attached cells and their total protein content at these time points in both non-cryopreserved and cryopreserved cultures are shown on Fig. 2, and the data show that a large majority of cells remain attached in culture post-thaw. The area of the monolayer of cells cryopreserved as a percentage of non-cryopreserved controls was $76 \pm 9\%$, and similarly the amount of total protein content was $72 \pm 13\%$ ($n = 15$ experiments, each performed in duplicate).

Intracellular GSH levels fell after thawing cryopreserved cells (Fig. 3). For example, 72 h post-cryopreservation intracellular GSH levels had declined to $46 \pm 8.1\%$ of non-cryopreserved controls (96 h non-cryopreserved cultures). In 96 h cultures which had not been cryopreserved $1.8 \pm 0.3 \text{nmol} \text{CDNB-GSH}$ were formed per minute ($n = 3$). Post-cryopreservation the hepatocytes formed $1.55 \pm 0.5 \text{nmol per minute at 72 h (n = 3)}$. The decrease in intracellular GSH levels was not sufficient to significantly compromise conjugation of CDNB under the conditions used. Increasing the equilibration time (times from 10 to 40 min, at 10 min intervals, were tested) of cells with the freezing medium prior to cryopreservation did not improve retention of GSH, and GSH levels declined after a 20 min equilibration period (results not shown). Incorporation of the glucose wash step caused a small, but insignificant improvement, in post-cryopreservation GSH levels (results not shown). The effect of adding various antioxidants to the cryoprotectant medium is shown on Fig. 4. The presence of both $\alpha$-tocopherol and ascorbic acid in combination proved most effective in limiting the loss of GSH in the cryopreserved cells, but interestingly their beneficial effect was lost when all the antioxidants were present in the freezing medium together.

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4. Discussion

Cryopreserving hepatocytes as monolayers offers enormous advantages in terms of effective retention of cell numbers post-thaw. The cells retain normal morphology, and when quantified in terms of the area of viable attached colonies approximately 75% of cells remain attached to the collagen coated plate (Stevenson et al., 2004). Watts and Grant (1996) showed that immediately post-thaw the monolayer hepatocyte membranes show signs of damage, but this is rapidly repaired. The loss of up to 50% of the intracellular GSH may be partly due to leakage through damaged membranes. The earliest measurement of GSH content made was at 24 h post-thaw; the rate of loss at earlier points is not known at present. GSH levels remained remarkably stable between 24 and 72 h both in non-cryopreserved and cryopreserved cultures. In the cryopreserved monolayers use of GSH must be minimal as they do not have the capacity to replete their stores by synthesis. They

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Fig. 1. CLSM images of CFDA and EtBr stained non-cryopreserved (left picture) and cryopreserved (right picture) rat hepatocytes. The pictures were taken at 96 h in non-cryopreserved cultures and 72 h post-cryopreservation. Scale bars are on the images.
may however, be able to re-distribute intracellular thiols to maintain sufficient intracellular GSH. Ascorbic acid and α-tocopherol prevented the loss of GSH in the cryopreserved monolayers to a large extent, and inclusion of these antioxidants in the freezing medium retained a level of GSH in the cryopreserved cells at 72 h post-thaw which was not significantly different from that in 96 h non-cryopreserved cultures. The conjugation of CDNB at a concentration of 50 μM was not compromised by the decreased availability of GSH in the cryopreserved monolayers. This
is in marked contrast to the situation in cryopreserved rat and human hepatocyte suspensions where the GSH conjugation of CDNB in intact cells was less than 10% of that in freshly isolated cells, and GSH was severely depleted (Sohlenius-Sternbeck and Schmidt, 2005).

There was a slight increase in the expression of GCL after cryopreservation. GSH levels exert an inhibitory feedback on the activity of GCL by binding to the glutamate site and another site on the enzyme (Lu, 1999). However, this action is at the level of the activity of the preformed enzyme not on gene expression. The lowered intracellular GSH is unlikely to be responsible for the increased expression. GCL is the rate limiting step of GSH biosynthesis. It is a dimer consisting of a heavy subunit which is the catalytically active part of the enzyme, and a regulatory light subunit (Anderson, 1998). In the present study the expression of the catalytic subunit was being measured. The gene expression of both subunits is increased by conditions which cause oxidative stress and activate NF-κB (Cai et al., 1997). A disturbance of the redox balance in the cells resulting in oxidative stress more than likely caused the increase in the amount of the GCL enzyme. This is supported by the improvement in GSH levels in the cells when $\alpha$-tocopherol and ascorbic acid were present as antioxidants. However, this increase could not resolve the deficit in GSH because there was no detectable GS present after cryopreservation of the cells. Detection of the normal levels of GS expression in the cells far exceeded the limits of detection of our analytical technique.

Fig. 4. GSH content of 72 h cryopreserved cultures frozen in media containing antioxidants. $\alpha$TA medium contains $\alpha$-tocopherol and ascorbic acid; D contains deferoxamine; SodCat contains catalase and superoxide dismutase and A contains all the antioxidants present together. Results are expressed as a percentage of the 96 h non-cryopreserved control cultures in terms of nmol GSH/ mg protein. Values are means ± SEM, $n = 3$.

Fig. 5. Expression of GCL (a) and GS (b) with time in monolayer cultures with (CP) and without cryopreservation. Results are the optical density of the bands on the immunoblot, and are means ± SEM, $n = 3$. 

![Graph](image1.png)  

![Graph](image2.png)
tions, E.J. Shanks and L.I. McLellan). Although there are no values available for GS expression in hepatocytes, these investigators estimate that in cell lines (Jurkat, HeLa and Cos cell lines) levels of GS are between 0.005 and 0.1% of total cell protein on the basis of western blots. Thus, a decrease in levels of GS expression to less than 10% of controls could have been readily detected in the present work. Less is known about the regulation of GS expression. The enzyme has two identical subunits, and is glycosylated. It is not induced by oxidative stress, nor inhibited by GSH (Lu, 1999). Expression of the enzyme was relatively stable in cultured hepatocytes, but absent post-cryopreservation. At present the reason for this is not known.

In conclusion, monolayers of hepatocytes post-cryopreservation in a freezing medium containing α-tocopherol and ascorbic acid retain 84% of the GSH levels compared with non-cryopreserved monolayer cultures. However, the absence of GS enzyme protein post-cryopreservation means there is no resynthesis possible. Many reports in the literature show that when GSH is depleted in hepatocytes the toxicity of reactive chemicals is exacerbated (Kanz et al., 2003; Chen et al., 2005; Kurebayashi and Ohno, 2006; Schauer et al., 2004). The cryopreserved monolayers may therefore be more susceptible to the toxicity of reactive intermediates, and any potential toxic effect of xenobiotics will be emphasised, leading to the possibility of false positive results of toxicity tests.

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References


