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PRELIMINARY COMMUNICATIONS

HEPATOCYTES IN PRIMARY CULTURE BECOME SUSCEPTIBLE TO PARACETAMOL INJURY AFTER DEPLETION OF GLUTATHIONE USING DL-BUTHIONINE-SR-SULPHOXIMINE (BSO).

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INTRODUCTION.

Primary cultures of hepatocytes are poorly susceptible to the toxic effects of paracetamol in comparison with suspended hepatocytes [1,2]. One possible reason for this tolerance of high paracetamol doses may be due to the high levels of glutathione in the attached cells. On the other hand the suspended hepatocytes die far more rapidly than cells *in vivo* and have abnormal permeability characteristics. DL-Buthionine-SR-sulphoximine (BSO) has been shown to be an effective inhibitor of γ-glutamylcysteine synthetase [3], an enzyme required for glutathione synthesis, without producing toxic effects *in vivo* or in cultured cells. We have shown that there is an increased susceptibility to paracetamol as measured by an increase in cytosolic enzyme leakage, in cells cultured with BSO and exposed to paracetamol for a short time, when compared with cells exposed to paracetamol alone.

MATERIALS AND METHODS.

Male wistar rats (180-250g) were given 0.1% phenobarbitone in their drinking water for 5 days. On the day before sacrifice the animals were given an oral dose of 20mg vitamin E in olive oil. Isolated hepatocytes were prepared by collagenase perfusion of the liver as previously described [4]. 2×10^6 cells were placed into 60mm diameter, Primaria, tissue culture plates (Falcon, Beckton Dickinson U.K.) at 37°C in an atmosphere of air containing 5% CO_2 at 100% humidity, in Williams Medium E (WME) supplemented with 10% (v/v) bovine foetal calf serum (FCS) (Gibco U.K.), 2mM L-glutamine and 50μg/ml gentamycin in a total volume of 2.5mls of medium. BSO (50μM) (Chemical Dynamics Corporation U.S.A.) was added for 2 hours. The medium was aspirated and replaced with one containing paracetamol (10mM) (Sigma Chemical Company U.K.), with or without BSO and CaEDTA (4mM) (Sinclair Pharmaceuticals U.K.) as required and exposure continued for 4 hours. The paracetamol and BSO were removed from the incubation medium and the primary cultures were reincubated in WME + 5%FCS with and without CaEDTA for a further 18 hours. At 24 hours LDH leakage was determined [5]. Cell number in the culture plate was ascertained from DNA measurement of the adherent cells [6] and glutathione levels measured using an enzymatic recycling assay [7].

RESULTS.

Alterations in glutathione levels of primary cultures of hepatocytes verus BSO concentration after 18 hours exposure are illustrated in Figure 1. It has been shown previously that 20μM BSO leads to a complete inhibition of γ-glutamylcysteine synthesis in an isolated enzyme system [3]. Our data show that a 20μM concentration of BSO produced a 37% reduction in the glutathione level after 18 hours incubation. At 50μM BSO there was a 70% reduction in glutathione level without any sign of toxicity. This concentration of BSO was adopted for further experiments.

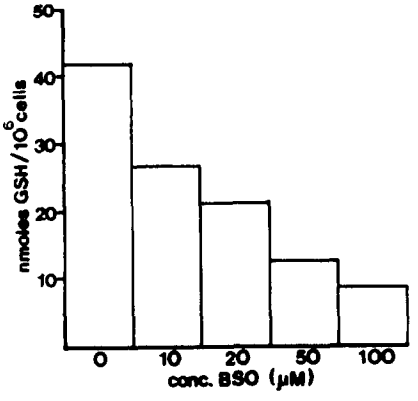

FIGURE 1. Alteration in glutathione levels of primary cultured hepatocytes from phenobarbitone treated rats after exposure to BSO for 18 hours. Isolated hepatocytes were plated at $2x10^6$ cells/plate in WME+10%FCS for 6 hours after which the medium was replaced with WME+5%FCS containing the required BSO concentration.

The primary cultures of hepatocytes were exposed briefly to paracetamol (10mM for 4 hours) with and without BSO. LDH leakage was then measured as shown in Table 1.

	LDH LEAKAGE, % OF TOTAL 6hrs.	24hrs.
EXPERIMENT 1		
CONTROL	7.3±2.1	35.8±10.5
PARACETAMOL	3.0±3.9	57.5± 9.7
BSO	8.9±3.5	31.4± 6.4
BSO + PARACETAMOL	3.9±5.7	74.1± 5.6
EXPERIMENT 2		
BSO + PARACETAMOL	7.1±2.2	56.9± 6.0
CaEDTA, BSO + PARACETAMOL	2.0±1.7	56.5± 9.0

TABLE 1. The effect of treatment from 0 to 6 hours with BSO and from 2 to 6 hours with paracetamol on LDH leakage at 6 and 24 hours in primary cultures of hepatocytes. Isolated hepatocytes were plated at $2x10^6$ cells/plate in WME+10% FCS ±BSO (50μM). The medium was then replaced after 2 hours with fresh medium containing paracetamol (10mM) as required. At 6 hours the BSO and paracetamol were removed and the medium replaced by WME+5%FCS. Results expressed as mean ± 1SD (n=6).

As can be seen the control group has a fairly high rate of leakage over the 24 hour period. However the paracetamol treated group showed a

significantly higher leakage than the control group. The cells differ markedly in that the paracetamol treated cultures did not form the adherent syncitial layers found in the control plates [4].

CaEDTA protects suspended hepatocytes from paracetamol injury but we found that 4mM CaEDTA was unable to arrest the LDH leakage induced by BSO and paracetamol. In other experiments we have shown that CaEDTA penetrates suspended hepatocytes but not into cultured cells.

When the glutathione levels for the cultures were measured at 24 hours (Figure 2) the control cell values were approximately 20% higher than those for the cultures which had been exposed to BSO for the initial 6 hours. The attached cells from the paracetamol and BSO treated cultures had GSH levels 75% lower than those of the control groups. CaEDTA had little effect on GSH levels. Evidently the injured cells cannot resynthesise GSH even though the medium (WME) contains 0.3mM cysteine and 0.1mM methionine.

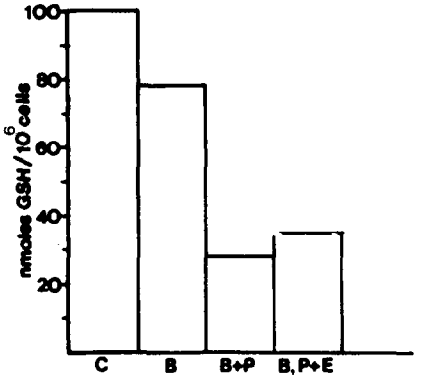

FIGURE 2. The effect of treatment to 6 hours with BSO and paracetamol on glutathione levels at 24 hours in primary cultures of hepatocytes. Isolated hepatocytes were plated in WME+10%FCS, with BSO (B) or without (C) for 2 hours. Paracetamol (P) and CaEDTA (E) were added to BSO treated cultures as required. At 6 hours both BSO and paracetamol were removed and the medium replaced by WME+5%FCS in all the plates.

DISCUSSION.

From these results it can be concluded that when primary cultures of hepatocytes are treated with BSO over a short 6 hour incubation and reincubated for a further 18 hours in the absence of the γ-glutamylcysteine inhibitor the concentration of glutathione can recover almost to its normal level. However when the primary cultures are plated with BSO and exposed to paracetamol from 2-6 hours and reincubated in control medium for a further 18 hours then both LDH leakage and loss of glutathione continue as the cell injury develops.

The slow developement of cell injury following a 4 hour exposure of the cells to paracetamol may be a more realistic model for the liver injury found after paracetamol overdose, than systems in which continous paracetamol exposure is used or which cells are killed in a short time. It may allow better investigation of the progress of cell injury and protection.

It has been shown that paracetamol alone causes depletion of glutathione in paracetamol treated hepatocytes [8] and when the cells are

incubated in a medium devoid of sulphur amino acids further resynthesis is prevented. In our model the paracetamol would appear to be reducing the resting glutathione level and the BSO also prevents resynthesis, causing a very rapid glutathioine depletion followed by deleterious effects on cell viability. Although CaEDTA penetrates and protects suspended hepatocytes against paracetamol toxicity [2] it is unable to protect the hepatocytes under the circumstances of attached culture where CaEDTA does not penetrate the cells.

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