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DYNAMIC ORGAN CULTURE OF PRECISION LIVER SLICES FOR IN VITRO TOXICOLOGY

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Summary

The lack of a reproducible method for the production of thin tissue slices has hindered the use of liver slices as an in vitro tool for hepatotoxicity studies. Fresh human, rat, and rabbit liver was processed using a mechanical slicer. With this instrument, precision (5% of thickness) liver slices in the submillimeter range could be produced at a rapid rate. Slices were prepared from fresh livers in chilled, oxygenated buffer to minimize trauma. Following incubation for up to 20 h in a dynamic organ culture system, histology of incubated slices suggested that 250 m precision-cut slices were optimum in regard to morphology relative to liver slices incubated under conventional organ culture conditions. Addition of bromobenzene to the culture showed time-dependent hepatotoxicity based on two classic parameters of cell degeneration. Histological evidence is presented which suggests the usefulness of this system for hepatotoxicity studies and the production of focal necrosis in vitro.

Introduction

In vitro systems employing cultured liver cells are finding widespread use in both descriptive and mechanistic studies of chemical toxicity (Grisham, 1979). The advantages which these have over classical methods, such as epidemiologic studies on human populations and whole-life studies on experimental animals, include decreased cost and time, and reduced numbers of animals (Bridges, 1976). In addition, mechanisms of action of chemical toxins are difficult to evaluate in vivo because of complicating secondary systemic effects and the difficulty of identifying short-lived toxic metabolites (Moldeus et al., 1978).

Biochemically fully active suspensions of isolated hepatocytes, prepared by enzymatic dissociation of the liver, are used extensively in such studies; however, there are certain disadvantages associated with their use including instability of enzymes in long-term experiments (Eckwall, 1980), lack of the liver cell heterogeneity found in vivo (Sweeney, 1983 and Hildebrand, 1983), and the rapid deterioration of mixed function oxygenase integrity (Guzelian et al., 1977).

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In an effort to provide a viable alternative to isolated hepatocyte cultures for toxicological studies, a rapid and simple organ culture system which uses precision cut mammalian liver slices has been developed. Fresh slices are produced in large quantities at a rapid rate under conditions which result in minimal tissue trauma (Krumdieck et al., 1980). For incubation, a novel dynamic organ culture procedure was used in which the advantages of roller culture (increased surface area, constant agitation of the media, and increased rate of gas exchange) have been combined with those of more conventional static organ culture (retention of architecture and consequent cell interactions, and maintenance of differentiated structure; Freshney, 1983) to yield a tissue preparation which appears to remain viable for at least 20 hours. With this system bromobenzene toxicity in mammalian liver has been successfully studied. The results suggest that this system may ultimately provide a simple and reliable alternative to existing in vitro tissue and/or cell culture systems for both the screening of toxins and the study of mechanisms of toxicity.

Methods

Chemicals. Bromobenzene was obtained from American Drug and Chemical Co. (Culver City, CA) and was dissolved in dimethyl sulfoxide (DMSO) [Eastman Kodak Co., Rochester, NY]. Phenobarbital sodium was obtained from Mallinckrodt, Inc. (St. Louis, MO). Waymouth's Medium MB 752/1 powder without phenol red or sodium bicarbonate was purchased from Gibco Laboratories (Grand Island, NY) as was fetal bovine serum. Gentamycin was purchased from Sigma Chemical Co. (St. Louis, MO). N-2-Hydroxyethyl piperazine-N^1-2-ethanesulfonic acid (HEPES) [Calbiochem, La Jolla, CA] was used to buffer the culture medium.

Biological Specimens. Human liver tissue was obtained fresh from either organ donors or as biopsy material from patients undergoing surgical resection. Animals included adult New Zealand white rabbits (Blue Ribbon Ranch, Tucson, AZ) and male Sprague-Dawley rats (Division of Animal Resources, University of Arizona, Tucson, AZ).

Slice Incubation Studies. Liver slices (200 ± 10 to 350 ± 15 μm) were prepared from freshly-isolated mammalian liver (human, rabbit and rat) on a modified version of the Krumdieck tissue slicer (Krumdieck et al., 1980) under cold Krebs bicarbonate buffer with constant aeration (95% O_2/5% CO_2). Slices were placed on the inside walls of stainless steel roller-equipped steel mesh cylinders (260 μm pore size; 1.2 cm I.D.), which were then placed in scintillation vials containing 2 ml of Waymouth's MB 752/1 media supplemented with 10% fetal calf serum and Gentamycin (84 μg/ml) [13]. Vials were capped with silastic rubber septa (300 μM)to allow gas exchange and placed horizontally on a vial rotator (Bel-Art Products, Pequannock, NJ). The entire rotator was placed in a Napco model 322 laboratory incubator (National Appliance Co., Portland, OR) set at 37°C. The vials were rotated at 10 rev/min and culture media replaced every 12 h. For histological evaluation and thickness determination, slices were fixed in 10% buffered neutral formalin and plastic embedded in glycol methacrylate (Dupont, Newtown, CT). Plastic sections were stained with hematoxylin and eosin (H & E) and examined by light microscopy.

Toxicity Studies. Male Sprague-Dawley rats (approximately 300-350 g) were induced with phenobarbital sodium according to the induction schedule reported by Maiorino et al. (1982). Slicing and incubation conditions were similar to the above with the exception that the vials were sealed with aluminum-coated vial caps and gassed every 2 h with O_2/CO_2 (95%/5%) immediately after delivery of bromobenzene to the media of individual culture vials. Stock bromobenzene solutions were prepared in DMSO and final DMSO levels were less than or equal to 1%. Following the appropriate incubation times, slice K^+ content and lactate dehydrogenase (LDH) activity in the media were determined as indices of bromobenzene toxicity (Gottschall et al., 1984 and Dankovic and Billings, 1984). Significant differences between control and treated slices were determined by Student's t-test with $p<.05$ as the cutoff for significance.

Results

Slice Consistency. Slices produced with the Krumdieck tissue slicer were evaluated for consistency by measuring thickness and wet weight. Slice thickness was measured in plastic-embedded slices to compare variations in thickness, thus an index of cutting precision, within individual slices and between different slices. By embedding the slices vertically in plastic, it was possible to produce longitudinal microtome sections which represented an entire slice. Using an ocular micrometer, twelve random measurements were made on each slice and the mean thicknesses compared. The average thickness of 6 consecutive slices cut in the 400 m range was 382 ± 19.7 μm (mean ± SD). The average wet weight of a series of non-consecutive rabbit liver slices (300μm thick, 15 mm diameter) was 65.8 ± 6.85 mg (n = 32).

Slice Incubation Studies. Histology of incubated liver slices indicate that slices remain more normal morphologically in the described culture system than they do in more conventional static systems of organ culture. When rat liver slices were incubated under conventional static organ culture conditions [e.g., Trowell culture (Campbell and Hales, 1971)], they developed a characteristic "banding" pattern suggestive of degenerative changes occurring in areas where gas and/or nutrient diffusion was limited (Fig. 1a). In contrast, rat liver slices incubated in roller culture did not demonstrate these apparent degenerative changes (Fig. 1b) at 20 h but instead maintained normal morphology with regard to cell architecture, nuclear integrity, and differential staining. When human liver slices were incubated using the same dynamic organ culture system (DOCS), they appeared to retain normal morphology for up to at least 20 h. Slices (200 μm) showed open sinusoids, consistent cellular architecture, intact nuclei, prominent nucleoli, and minimal vacuolization (See Fig. 2).

Toxicity Studies

Lactate Dehydrogenase Leakage. LDH leakage from phenobarbital-induced rat liver slices exposed to bromobenzene (1 mM) in dynamic organ culture was time-dependent. While leakage was not significantly different from control at 2 h, by 4 h LDH leakage was approximately 220% ($p<.05$) that of control and at 6 h, LDH leakage was 290% of control ($p<.05$) [see Fig. 3]. In rat liver slices from non-induced animals, incubation with bromobenzene produced no significant increases in LDH release (Fig. 4). This finding emphasizes the requirement that bromobenzene be oxidatively metabolized via cytochrome P-450 mixed function oxygenases in order to produce a toxic metabolite and suggests that slices incubated in the DOCS maintain xenobiotic metabolising activity for at least 6 h.

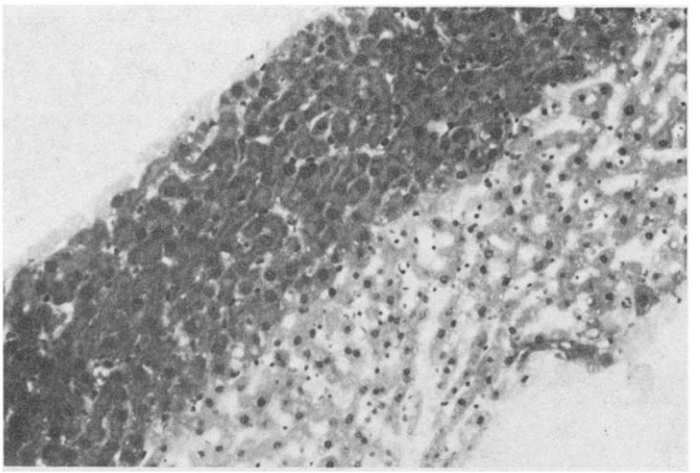

Fig. 1a

Photomicrograph demonstrating the deterioration of liver cells at the media/slice interface at 20 h in rat liver slices (250 μm) incubated in Trowell (static) culture. Note the lower half of the slice which has undergone severe degeneration. (H- and E-stained section of plastic-embedded slice x 100).

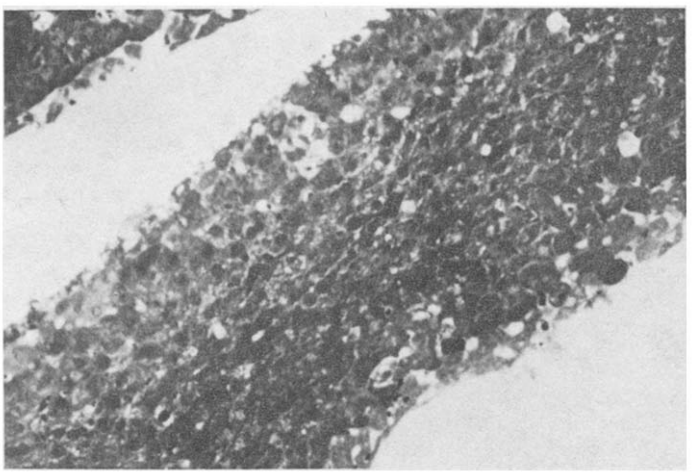

Fig. 1b

Photomicrograph representative of rat liver slices (250 μm) incubated in a dynamic organ culture system (DOCS) where there is constant gas exchange; note the overall healthier appearance and absence of degenerative "banding" as seen in Figure 1a at 20 h. (H- and E-stained section of plastic-embedded slice x 100).

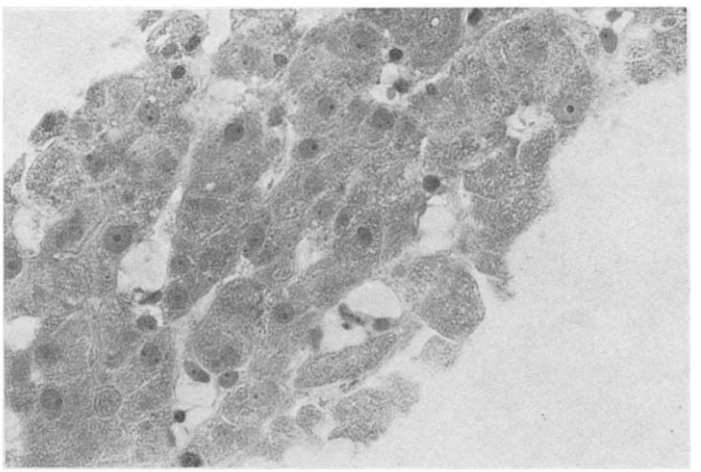

Fig. 2

Photomicrograph of human liver slice (200 μm) incubated in dynamic organ culture system (DOCS) for 20 h. Note the maintenance of cell architecture, nuclear membrane, and prominent nucleoli; also, note lack of significant vacuolization. (H- and E-stained section of plastic-embedded slice x 400).

Slice K^+ Content. The content of K^+ remaining in cultured cells has been widely employed as an index of cell viability. Hence, leakage of K^+ from cells would indicate toxic insult or cell degeneration. In bromobenzene-treated rat liver slices prepared from phenobarbital-induced animals time-dependent kinetics of slice K^+ loss similar to LDH leakage described above were observed (Fig. 5). However, by 4 h loss of slice K^+ appeared to be near maximal since it was 28% and 26% of control by 4 h ($p<.05$) and 6h ($p<.001$), respectively. In rat liver slices from non-induced animals, slice K^+ content did not significantly differ between bromobenzene-treated and control animals by 6 h (Fig. 6).

In Vitro Centrolobular Necrosis. Rat liver slices were prepared from phenobarbital-induced animals and exposed to bromobenzene (1 mM) in dynamic organ culture. Slices were fixed, embedded and longitudinal sections prepared for histological analysis. H- and E-stained sections of slices incubated for 4 h demonstrated frank centrolobular degeneration (Fig. 7) in contrast to either normal, healthy-appearing periportal areas from the same slice or centrolobular areas from non-intoxicated control slices. The time course of the observed histological damage appears to parallel the biochemical changes described above.

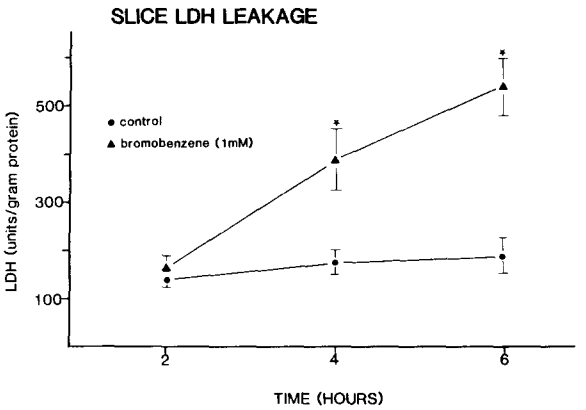

Fig. 3

Bromobenzene toxicity in phenobarbital-induced rat liver slices (300 μm) in dynamic organ culture (DOCS) as indicated by leakage of lactate dehydrogenase (LDH). Each point represents the mean ± SEM of 3 animals (6 slices). Bromobenzene concentration was 1 mM and dimethyl sulfoxide (DMSO) was used as the vehicle control. Note the significant changes in LDH leakage by 4 h when liver slices from phenobarbital-induced rats were exposed to bromobenzene (*$p<.05$).

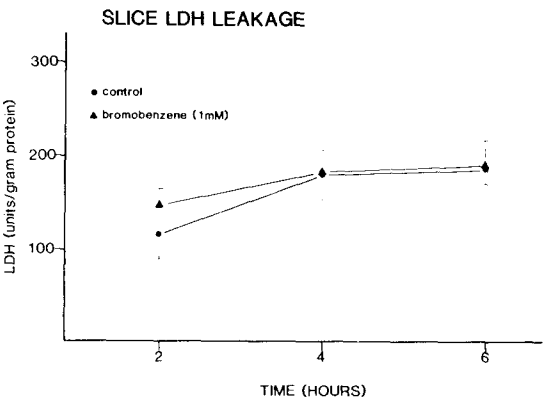

Fig. 4

Apparent lack of toxicity to bromobenzene (1 mM) in control (non-induced) rat liver slices in DOCS as determined by LDH leakage. Note that at 6 h, there was no significant difference in LDH leakage between treated and non-treated slices (n = 3).

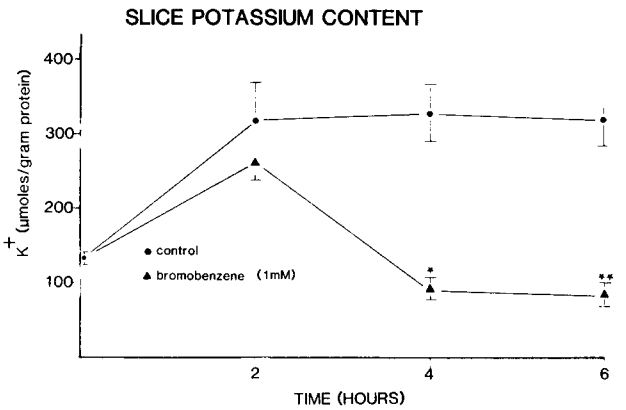

Fig. 5

Content of K^+ remaining in rat liver slices prepared from phenobarbital-induced rats. Slices were incubated in DOCS and exposed to bromobenzene (1 mM) over 6 h. Note the significant loss of slice K^+ by 4 h (*$p<.05$) and 6 h (**$p<.001$) [n = 3].

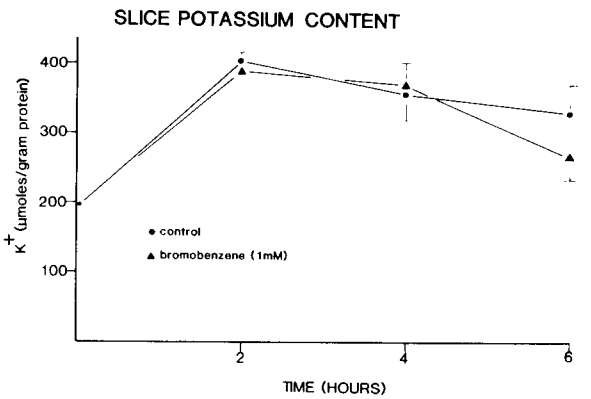

Fig. 6

K^+ content of non-induced (control) rat liver slices following addition of bromobenzene (1 mM) to culture medium. Note the lack of effect of bromobenzene in causing K^+ release in non-induced rat liver slices in DOCS (n = 3).

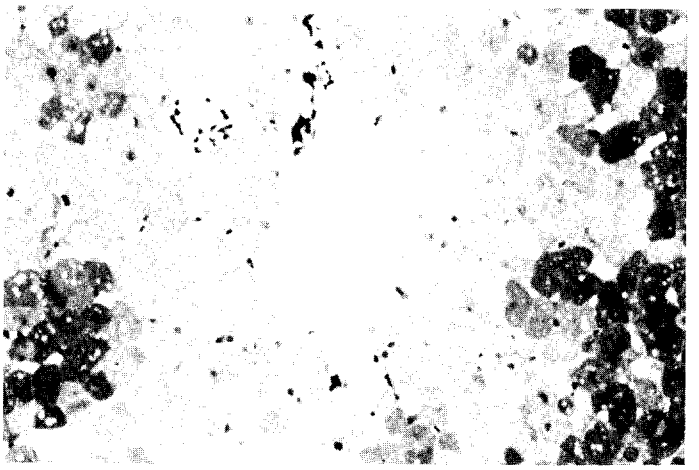

Fig. 7

Photomicrograph of centrolobular area from rat liver slice (350 μm) of a phenobarbital-induced animal exposed to bromobenzene (1 mM) for 4 h in dynamic organ culture. Note the loss of cellularity, decreased cytosolic staining, loss of architecture and nuclear degeneration in cells immediately surrounding central vein. (H- and E-stained section of plastic-embedded slice x 400).

Discussion

While isolated perfused liver and isolated hepatocyte suspensions provide valuable tools for the study of hepatic physiology and hepatotoxicity respectively, there is presently no satisfactory in vitro preparation to bridge the gap between these systems (Sweeney, 1983). Such a preparation should be at the sub-organ level, and it should maintain the functional capacity of isolated hepatocytes. Furthermore, the "functional heterogeneity", characteristic of the liver's acinar organization, should also be maintained. The system described herein, which utilizes precision liver slices, appears to fulfill these criteria. It has been demonstrated that these slices are consistent enough, with regard to thickness and surface area, to be used as individual experimental units. Additionally, the slices combine tissue superstructure and cell-cell interactions as present in perfused tissue with the high sample-to-sample reproducibility of cell suspensions. By combining dynamic culture conditions with organ culture of thin liver slices, the problem of limited oxygen diffusion associated with earlier organ culture methods of adult mammalian liver (Cambell and Hales, 1971) appears to have been overcome.

Currently this system is being used for the study of hepatotoxins. Since many liver slices can be prepared from a single animal liver, it will be possible to screen a number of potential toxins using fewer animals. In particular, bromobenzene toxicity has been studied using lactate dehydrogenase (LDH) leakage, slice potassium content and histology as endpoints. The results indicate that this system is at least as sensitive as isolated hepatocyte suspensions for the study of bromobenzene toxicity (Dankovic and

Billings, 1984). In addition, histological changes in liver which are normally associated with bromobenzene toxicity in vivo (e.g., centrolobular necrosis), were reproduced using this in vitro system of incubated liver slices.

It is anticipated that dynamic organ culture of precision cut liver slices will provide a valuable addition to current in vitro methods for investigations of potentially hepatotoxic compounds.

Acknowledgements

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