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Extended rat liver slice survival and stability monitored using clinical biomarkers☆

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Abstract

Precision-cut liver slices are reportedly limited as toxicity models by their short half-life in culture. We used traditional clinical chemistry biomarkers and histology to assess a newer procedure for improved liver slice maintenance. Slices from Sprague–Dawley rat livers were well maintained in a roller culture system for up to 10 days based on protein content (60–70% or higher of initial values) and biomarker retention and verified by histological examination of the tissues showing morphological integrity and viability of hepatocyte and biliary regions. Exposure of the slices to geldanamycin (GEL) resulted in loss of slice LDH and transaminase content, with associated depression in ALP and GGT levels and elevated bilirubin, indicating that GEL affects both cell types as occurs in vivo with this hepatobiliary toxicant. Thus, we conclude that liver slices merit further investigation as a general model for chronic as well as acute toxicity studies.

Keywords: Precision-cut rat liver slices; Hepatobiliary toxicity; Geldanamycin; In vitro systems

The value of toxicological data from precision-cut liver slices for predicting and studying clinically relevant effects of candidate drug compounds has been limited in large part by the slices' short survival time in culture (48–72 h) [1,2]. Fisher et al. [3] have reported preparing rat liver slices that can survive for up to 5 days. More recently, success in extending rat liver slice cultures to 4 days and human liver slices for 7 days has also been reported [4–6]. The capability to culture organ slices for more extended periods may make possible the study of chronic as well as acute effects of chemicals and drugs at more physiologically relevant exposures [2]. Consequently, we examined the possibility of extending the culture period by taking advantage of recent refinements in procedure that also prevent necrosis at the contact points with the support mesh [5]. This included (1) flushing the liver and slicing the cores in cold UW (University of Wisconsin) solution, (2) loading the slices onto cellulose-ester filters placed on the mesh inside the inserts in a dynamic roller culture system rather than directly in contact with the titanium mesh or Teflon screen, and (3) culturing in a defined medium based on Waymouth's MB 752/1 under a high O_2 content atmosphere. Using these procedures and monitoring with traditional clinical chemistry biomarkers and histology, we demonstrated for the first time that rat liver slices can retain their integrity and viability for at least 10 days in culture. Investigations with a known hepatobiliary toxicant, geldanamycin (GEL), produced biochemical and morphological deficits in both hepatocytes and biliary cells in this model system, consistent with its target cell effects in vivo [7].

Materials and methods

Animals, supplies, and equipment. Young adult male (~300 g) and female (~270 g) Sprague–Dawley rats were purchased from Charles

☆ *Abbreviations:* UW, University of Wisconsin (solution); GEL, geldanamycin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Bili-T, total bilirubin; GGT, γ-glutamyltransferase; LDH, lactate dehydrogenase; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff's reagent; CV, percent coefficient of variance.

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River (Hollister, CA). Sodium pyruvate was purchased from JT Baker (Phillipsburg, NJ). Glutathione was purchased from EM Science (Gibbstown, NJ). Bovine serum albumin (BSA) was purchased from ICN Pharmaceuticals (Aurora, OH). Waymouth's MB 752/1 basal medium (#078-5105EL), Ca^+- and Mg^+-free phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), Glutamax I, and antibiotic–antimycotic solution were purchased from Invitrogen/Gibco (Carlsbad, CA). Viaspan (Belzer—University of Wisconsin (UW) cold storage solution) was purchased through Fisher Scientific (Pittsburgh, PA). All other chemical supplies were purchased from Sigma (St. Louis, MO). The tissue coring press and titanium inserts were purchased from Vitron (Tucson, AZ). HATF (0.45-μm surfactant-free hydroanalysis mixed cellulose ester triton-free) filter paper used for slice placement was purchased from Millipore (Bedford, MA). Low background, glass scintillation vials were purchased from Research Products International (Mt. Prospect, IL). The TC-8 roller drum unit was purchased from New Brunswick Scientific (Edison, NJ). GEL (99% purity) was obtained from the NCI Repository (McKesson Biosciences, Rockville, MD).

Liver slice preparation. All experimental work was conducted at SRI International except as otherwise stated. Rats were anesthetized with sodium pentobarbital (35–45 mg/kg). Once unconscious, the tail-vein was injected with 200 U heparin in physiological saline, and the animals were placed in a sterile biosafety hood, where all further work was done aseptically. After exposure of the abdominal cavity, the portal vein was perfused with ice-cold UW solution supplemented with 3 mM glutathione, 2 mM Glutamax I, 1× antibiotic–antimycotic solution, 30 μM L-ascorbic acid, 2 mM sodium pyruvate, 1 μM corticosterone, and 100 nM insulin (human). Once all of the lobes had been flushed clear of blood, the liver was quickly removed, and the lobes were separated with a scalpel and cored into 8-mm-diameter cylinders. The cores were then sliced in supplemented UW solution into 220- to 250-μm-thick discs, using a Krumdieck slicer (Alabama Research and Development, Munford AL). Only the most uniform-shaped slices were selected for experiments; each containing approximately 3 mg protein. The slices were gently placed onto precut HATF paper already in position inside the titanium inserts, using a P1000 pipetter and a sterile, cut P1000 tip. Minor adjustments in slice position were made using fine tweezers. All slice manipulations were done in ice-cold supplemented UW solution.

Slice preincubation/culture. Once slices were in place on the filter paper, the titanium inserts were placed into sterile scintillation vials each containing 1.7 ml culture medium (Waymouth's MB 752/1 basal medium containing per ml: 2 mg BSA, 0.084 mg gentamicin sulfate, 5 μg oleic acid, 5 μg linoleic acid, 0.5 μg DL-α-tocopherol, 7.9 μg D-thyroxine, ITS (5 μg insulin, 5 μg transferrin, and 5 ng selenium), 288 ng testosterone, 272 ng β-17estradiol, 39.3 ng dexamethasone, 30 ng glucagon, 0.02 U insulin, and 0.2 μmol L-2-phosphate ascorbic acid) [8]. Sodium pyruvate (2 mM) was also added to the medium in the majority of experiments. The vials were then capped with presterilized open-end caps containing foam inserts, each held in place by a hole-punched, Teflon liner to allow gas exchange with the external atmosphere. The capped vials were placed in the roller drum inside a humidified incubator at 37 °C under a 70% O_2/25% N_2/5% CO_2 atmosphere, and the drum was rotated at 6–7 rpm. Following equilibration for 2.5–3.0 h, the zero time point group was harvested and the remaining inserts were transferred to new scintillation vials containing the respective prewarmed media with or without GEL at predesignated concentrations in a biosafety hood. The slice medium in each vial was replaced with fresh medium daily until the slices were harvested, and the collected media were stored at 4 °C until later biochemical analysis in conjunction with the respective slices. The new vials were repositioned on the roller system in the incubator and the incubation was continued under the above conditions until all groups were harvested. Each group had 4 replicate vials, three for biomarker analysis and the fourth for histology and morphological examination.

Biomarker analyses. At the indicated times, slices plus filter paper were removed from their titanium inserts with tweezers and rinsed in PBS by briefly submerging the slice (still on its HATF paper) into a vessel containing ~20 ml PBS. Again with tweezers, each slice was transferred into a 1.5-ml Eppendorf tube containing 0.5 ml PBS + 0.5% Triton X-100 on ice. The slices were then homogenized and briefly sonicated at ice-cold temperatures. The resulting lysates were centrifuged in a Beckman Coulter Microfuge 18 centrifuge at 9000*g* for 5 min to remove particulate matter. The resulting supernatants were stored at 4 °C until the experiment was complete and then sent on ice with the corresponding media by courier to Quality Clinical Labs (Mountain View, CA) for analysis on a Hitachi 911 clinical analyzer. Biomarker levels measured from slice lysates taken within the first 24 h and analyzed periodically thereafter were stable at 4 °C for a minimum of 18 days. Cell-specific biomarkers analyzed included AST (aspartate aminotransferase) and ALT (alanine aminotransferase) for liver parenchyma and ALP (alkaline phosphatase), bili-T (total bilirubin), and GGT (γ-glutamyltransferase) for biliary function; LDH (lactate dehydrogenase) was included as a general cytotoxicity indicator [2,3]. A small fraction of the lysate was retained for protein analysis using the Pierce BCA protein assay kit (VWR International, West Chester, PA) and BSA standards in PBS + 0.5% Triton X-100.

Histology. Slices for morphological examination (one vial/group) were removed from the inserts while still on the filter paper and briefly rinsed in PBS. A small square of lens paper was then pre-wetted in 10% buffered formalin and placed over the open face of each slice, and this stack was placed between two foam inserts. The entire "sandwich" was placed in a histological cassette and fixed in 10% buffered formalin. Slices to be evaluated for glycogen content were fixed in 95% ethanol. The cassettes were sent by courier to Biopathology Sciences Medical Corporation (South San Francisco, CA) for paraffin embedding and H&E (hematoxylin and eosin) and PAS (periodic acid Schiff–glycogen-specific) staining. Slice sections were examined at SRI by Dr. David Fairchild, a board-certified veterinary pathologist.

Statistics. A two-way ANOVA with replication was done using an Excel spreadsheet. Treatment groups were compared to the respective control group. Values were considered significantly different if $P < 0.05$.

Results and discussion

In initial experiments with the modified method we compared supplemented DMEM and variants of Waymouth's 752/1 media. We found that the defined culture medium based on Waymouth's MB 752/1 developed by Green et al. [8] with or without added sodium pyruvate performed slightly better over 5-day incubations. Previous investigators found enriched Waymouth's/bicarbonate medium to keep slices viable for up to 5 days based on K^+ retention, protein synthesis/secretion, and LDH release [3]. We chose to use the supplemented Waymouth's medium with sodium pyruvate in our experiments, in accordance with Toutain et al. [9]. We also chose to use clinical chemistry biomarkers that provide useful information regarding the presence and severity of both hepatocellular and hepatobiliary injury or impairment of liver function, in order to monitor tissue stability over time, rather than general cytotoxicity indicators [10].

The results for the clinical chemistry parameters we used are summarized in Table 1. The analytical data

Table 1
Slice biomarker changes in 5 day incubations

Parameter[a]	Average value[b]				% Change 0 to 5
	Day = 0	1	3	5	
AST	4600	4030	4060	3820	−17
ALT	880	752	684	688	−22
ALP	17.2	68.4	155.0	194.0	1030
Bili-T	0.16	0.20	0.26	0.20	25
GGT	4.4	4.0	6.8	12.8	190
LDH	4080	3290	4880	5240	28
Protein	3.0	2.7	2.5	2.4	−20

[a] Values are expressed for enzymes in U/L/mg slice protein, for total bilirubin (bili-T) as mg/dl/mg slice protein, and for protein as mg/slice.
[b] Data are means of 5 experiments for enzymes and bili-T and of 7 experiments for slice protein. CV (all values) = 14%.

show that functional capacity was generally well maintained over a 5-day period. Slice protein content and hepatocytic markers AST and ALT were modestly lower (17–22%) relative to initial levels on Day 0 (immediately following preincubation). In contrast, slice levels of the biliary markers ALP and GGT increased appreciably (11- and 3-fold, respectively) and bilirubin and LDH likewise increased but to a much lesser extent (~25–28%) during the same period. These observations were considered to indicate cell proliferation and repair in the tissues and are consistent with those of others who found morphological evidence of stellate cell and neobiliary duct proliferation beginning 48 h after slice preparation and culture [5]. Our data show that this process is associated with time course differences in biomarker behavior, with bilirubin and ALP levels increasing and GGT and LDH decreasing on Day 1, all four parameters increasing on Day 3, and all but bilirubin continuing to increase through Day 5 in our experiments. These changes suggest that the injury/repair process is complex and involves, in part, biliary function disruption as well as surface cell damage due to the slicing procedure.

Slices incubated concurrently under the same experimental conditions for 5 days were sectioned and examined microscopically; representative photomicrographs from Day 0 and Day 5 samples are exhibited in Fig. 1. Histological evaluation indicated that hepatocytes and biliary regions in the tissues were >90% viable; glycogen levels were likewise estimated to be >90%. The capacity of the cells to accumulate glycogen with glucagon present in the medium supports the vitality and integrity of the liver slices through Day 5.

Slices from a male and a female Sprague–Dawley rat were incubated for up to 10 days. Protein content in each preparation was reasonably well maintained (60% and 70% of Day 0 values for male and female slices, respectively), and no consistent, pronounced differences were seen in other parameters that could be ascribed clearly to the sex. Data on ALT and ALP levels are shown in Fig. 2. The time course changes, as measured using the clinical parameters shown (as well as those not shown), indicate results similar to those in Table 1. Microscopic observations of slices on Day 10 (Fig. 3) likewise showed that liver parenchymal architecture was largely intact and most of the hepatocytes and biliary epithelium were viable. The viable hepatocytes exhibited marked glycogen deposition as evidenced by clear cytoplasm on H&E staining and positive PAS staining.

To assess whether hepatobiliary agents affected both hepatocytes and biliary cells in rat slice incubations, we tested GEL in our experimental system for up to 5 days. No prominent changes in slice biomarker levels were seen at 24 h after exposure to 0.10, 1.0, or 10 μM GEL

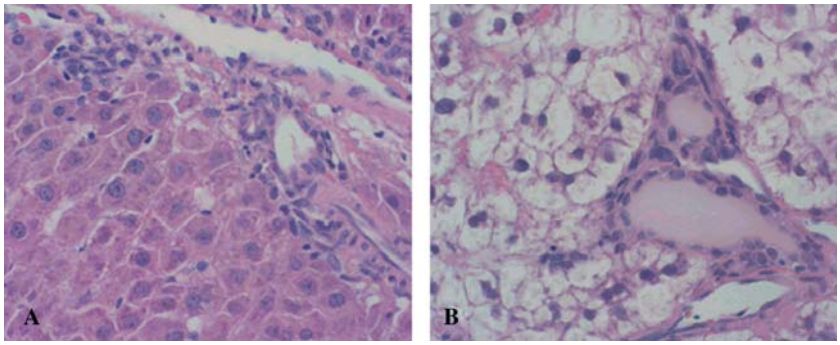

Fig. 1. Photomicrographs of liver slice transverse sections on Day 0 (A) after equilibration at 37 °C in the roller drum incubation system and on Day 5 (B) after daily replacement of culture medium with fresh medium (see Materials and methods). Magnification, 139×.

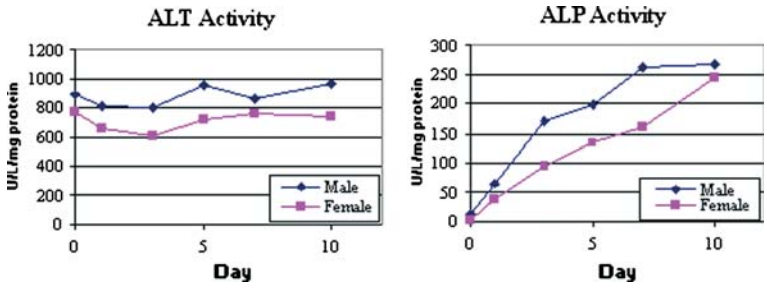

Fig. 2. ALT and ALP content in male and female Sprague–Dawley rat liver slices incubated for 10 days.

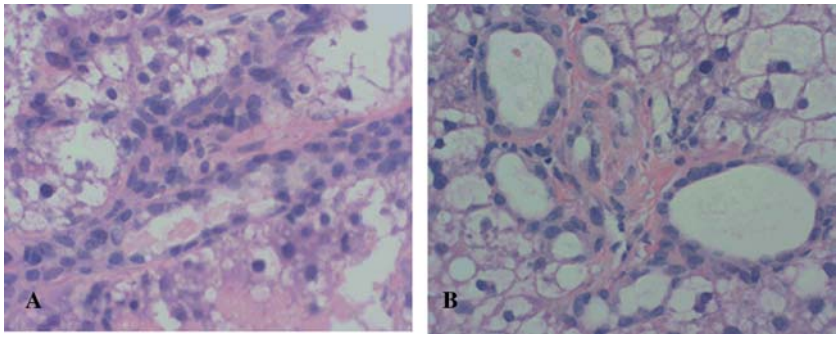

Fig. 3. Photomicrographs of transverse sections of male (A) and female (B) liver slices on Day 10 after daily replacement of the culture medium with fresh medium (see Materials and methods). Magnification, 139×.

except for increased bilirubin elevation at the higher concentrations. After incubation for 72 h or longer, all enzyme parameters were lowered in an essentially concentration-dependent manner and bilirubin remained higher relative to control (no GEL) levels. Fig. 4 shows values for the most significantly altered parameters: ALP, LDH, and GGT on Day 3. ALP depression was the most sensitive change, followed by LDH and GGT, and then ALT; AST was the least sensitive change and slice bilirubin remained elevated at slightly higher levels than on Day 1 (data not shown). Loss of ALT, AST, and LDH was accompanied by equivalent increases in these parameters in medium; however, the sensitivity of the analytical method did not allow corresponding measurement of ALP, GGT, and bilirubin. Morphologically, both hepatocytes and biliary epithelia showed decreased viability with periportal hepatocytes exhibiting massive necrosis of hepatic lobules. In intact animals, GEL produced generally similar results, namely, necrosis of both hepatocytes and bile ducts and elevations in serum ALT and AST in response to the liver injury [7]. These results were opposite to those observed with 6-mercaptopurine, a strong hepatotoxicant [11], in preliminary studies, as would be expected for the model system to respond correctly [12]. Our laboratory is currently engaged in efforts to elucidate the events oc-

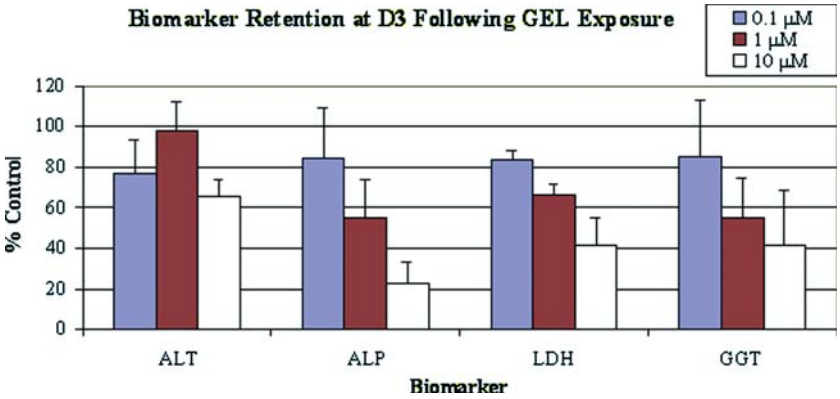

Fig. 4. GEL-induced slice biomarker changes after 3 days of continuous exposure.

curring in control slices over time and the mechanism of action of GEL in vitro relative to in vivo.

Our results essentially concur with those of other investigators [3,6] regarding the much longer viability of liver slices in culture than prevailing opinion seems to recognize. We affirmed the observations of Fisher et al. [3] in rat slices that the percentage of LDH and ALT released to the medium on Day 5 remains low and that longer incubation times should be possible under aseptic experimental conditions. The most notable differences between their approach and most others and ours were the decision here to use a battery of biomarkers for cell-specific, clinically relevant effects rather than general cytotoxicity and to monitor the stability of the slice parameters over time, as well as viability. With that approach, we discovered that the biochemical parameters associated with biliary function changed profoundly over time, indicating that the slices continue to undergo adaptation and repair well after 24 h, as noted previously [5], and on a similar timescale to what occurs in vivo in response to chemical-induced injury [13,14]. The effect of the toxicants we have studied to date appears to be superimposed upon or augment these background changes and be distinguishable from them such that the system may be useful as an assay of hepatobiliary toxicity. Further studies are needed to clarify this possibility.

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