# Cytoprotective influence of ZVAD-fmk and glycine on gel-entrapped rat hepatocytes in a bioartificial liver

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***Background.*** *This study was designed to determine if an anti-necrotic compound, glycine, and/or an anti-apoptotic agent, ZVAD-fmk, improved the viability and function of hepatocytes in a bioartificial liver.*

***Methods.*** *Isolated rat hepatocytes were entrapped in collagen gel (1.0 –10.0 × $10^6$ cells/mL) and cultured in serum-free medium (1:10 ratio of gel:media) supplemented with glycine alone, ZVAD-fmk alone, or glycine and ZVAD-fmk. The cytoprotective effects of glycine and ZVAD-fmk on gel-entrapped rat hepatocytes (GERH) were determined after anoxic exposure (0 – 20 hours). Cell functionality (measured by urea production), cell viability (quantitated by vital staining with fluorescein diacetate:ethidium bromide [FDA:EB]), and the mechanism of cell death (verified by electron microscopy and DNA fragmentation studies) were determined for each condition.*

***Results*** *The viability of GERH declined gradually and then stabilized 12 hours after hepatocyte isolation. The rate of urea production by GERH was directly proportional to the number of viable hepatocytes. Apoptotic death predominated at low cell density, and necrotic cell death became significant at high cell density. Hepatocyte necrosis became more significant after exposure to longer periods of anoxia (4, 8, 12, and 20 hours). ZVAD-fmk provided dose-dependent cytoprotection to GERH with an optimum benefit at a concentration of 60 μmol/L. After anoxic exposure or under high cell density culture, glycine demonstrated a maximum benefit of inhibiting necrosis at a concentration of 3 mmol/L. The beneficial effects of glycine and ZVAD-fmk were additive.*

***Conclusions.*** *The metabolic activity of a hepatocyte bioartificial liver may benefit from the use of cytoprotective agents such as ZVAD-fmk and glycine. (Surgery 2000;127:447-55.)*

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A NUMBER OF LIVER-ASSIST DEVICES have been developed, including the extracorporeal use of isolated mammalian hepatocytes in a bioartificial liver (BAL).[1] Criteria for successful application of a BAL include bridging patients to liver transplantation and avoidance of liver transplantation in cases of reversible hepatic failure.[2-4] In order to achieve these goals, hepatocytes in the BAL provide metabolic functions to the patient in liver failure. The extent to which these goals can be achieved is dependent on the number of viable and metabolically active hepatocytes in the device. Unfortunately, hepatocyte viability declines over time after isolation, and this decline limits the effectiveness and duration of BAL therapy.

Several BAL systems have been designed to maintain hepatocyte viability ex vivo. One such BAL system involves the entrapment of hepatocytes in cylindrical collagen gels located in the fibers of a hollow fiber bioreactor.[5-7] An in vitro model of this BAL system using gel-entrapped rat hepatocytes (GERH) has been developed for static culture experimentation and for the study of the mechanisms of hepatocyte death.[8,9] The in vitro model avoids the expenses of large-scale testing, and multiple experiments can be performed simultaneously. Potential immune interactions, present with in vivo models of BAL testing,[10] are avoided with in vitro testing.

Our recent work has shown that cell death of

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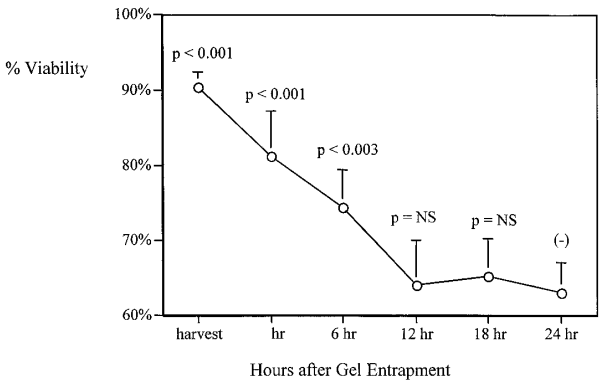


**Fig 1.** Viability profile of GERH during initial 24 hours of culture. Viability determined by FDA:EB staining. Cell density of $2.5 \times 10^6$ cells/mL of gel was used in this example. Data points *(open circles)* are the average viability of 8 gels and > 100 cells counted per gel. Error bars represent standard deviation. Viability at 24 hours was used for comparison and determination of *P* value (unpaired *t* test).

freshly isolated GERH occurs by apoptosis and necrosis.[11] We were therefore interested to see if ZVAD-fmk, a tripeptide with inhibitory properties against caspases and apoptosis,[12] or glycine, an anti-necrosis agent,[13-15] had a beneficial effect during culture conditions of relevance to a BAL. The specific aims of this study were to answer the following questions about glycine and ZVAD-fmk using GERH: (1) Do cytoprotective agents ZVAD-fmk or glycine improve the viability and function of GERH? (2) Do these cytoprotective agents have a beneficial effect under conditions of anoxia or high cell density? These questions are important to improve our understanding of cell death in the BAL and to reduce ischemic injury, which may develop in the BAL if hepatocytes are cultured at high cell density or under limited oxygen supply.

## METHODS

**Hepatocyte isolation and gel entrapment.** Rat hepatocytes were harvested from 4- to 6-week-old male Sprague-Dawley rats, weighing 200-250 g, by a 2-step, in situ collagenase perfusion technique modified from the method described by Seglen.[16] Briefly, after intraperitoneal pentobarbital injection (10 mg/100 gm body weight) and portal vein cannulation, in vivo perfusion (25 mL/min × 12 min) was performed with a calcium-free hydroxyethylpiperazine-ethanesulfonic acid (HEPES) buffered solution (143 mmol/L NaCl, 6.7 mmol/L KCl, 10 mmol/L HEPES, 100 mg/dL ethylene glycol-bis-aminoethyl ether, pH 7.4), and then perfused (20 mL/min × 10 min) with a second HEPES buffered solution (67 mmol/L NaCl, 6.7 mmol/L KCl, 4.8 mmol/L HEPES, 1.0 g/dL fatty acid free bovine albumin, pH 7.6) containing 0.05% collagenase D (clostridiopeptidase A, Boehringer Mannheim Corporation, Indianapolis, Ind). The liver was then removed and placed in a Petri dish containing 5°C William's E medium (Life Technologies, Grand Island, NY) supplemented with 5% calf serum, 2 mmol/L L-glutamine, 15 mmol/L HEPES, 1.5 mg/L insulin, 10,000 U/L penicillin G, and 100 mg/L streptomycin sulfate. The liver capsule was peeled back from all lobes, and the liver was gently combed to isolate and suspend hepatocytes in solution. The hepatocytes were gauze-filtered (110 μm) and washed (centrifuged at 50*g* for 1 minute and resuspended) 3 times before final suspension in William's E medium. Rat harvests yielded from $3.0\text{-}5.0 \times 10^8$ hepatocytes with an average viability of 90% as determined by trypan blue exclusion.

Gel entrapment was performed by first centrifuging the hepatocyte suspension to form a soft pellet. Supernatant was removed, and hepatocytes were suspended in a protein solution (3:1 mixture of type 1 collagen [Vitrogen 100; Collagen Corporation, Palo Alto, Calif] and 4-fold concentrated William's E medium supplemented with 1.7 mg/L insulin, 40,000 U/L penicillin G, 400 mg/L streptomycin sulfate, pH 7.6). The hepatocyte suspension (0.1 mL) was injected into sterile silicone tubing (0.64-mm internal diameter × 1.2-mm external diameter × 30-cm length, Dow Corning, Midland, Mich), and incubated at 37°C for 10 minutes to accelerate gel formation. Cells were entrapped in gel at low ($1.0 \times 10^6$ cell/mL), intermediate ($2.5\text{–}5.0 \times 10^6$ cell/mL), or high ($10.0 \times 10^6$ cell/mL) cell density.

After 10 minutes of incubation, control gels were transferred to 1.0 mL of serum-free culture media (William's E medium supplemented with 5 μg/L epidermal growth factor, 200 U/L insulin, 1 mg/L glucagon, 5mg/L transferrin, 0.5 g/L albumin, 5 mg/L linoleic acid, 1 μmol/L dexamethasone, 6.25 μg/L selenium, 40,000 U/L penicillin G, 400 mg/L streptomycin sulfate, and 15 mmol/L HEPES). Gels in the experimental groups were placed in serum-free media supplemented with 0-3 mmol/L glycine (Sigma Chemicals, St Louis, Mo) and/or 0-120 mol/L ZVAD-fmk (Enzyme System Products, Livermore, Calif). Stock ZVAD-fmk (20 mmol/L) was prepared in dimethyl sulfoxide (DMSO). Culture medium of control wells was supplemented with a final concentration of 0.2% DMSO, similar to the DMSO content of ZVAD-fmk containing wells.

Experiments were performed in duplicate (2 rats per experiment) with 6 to 12 gels per group.

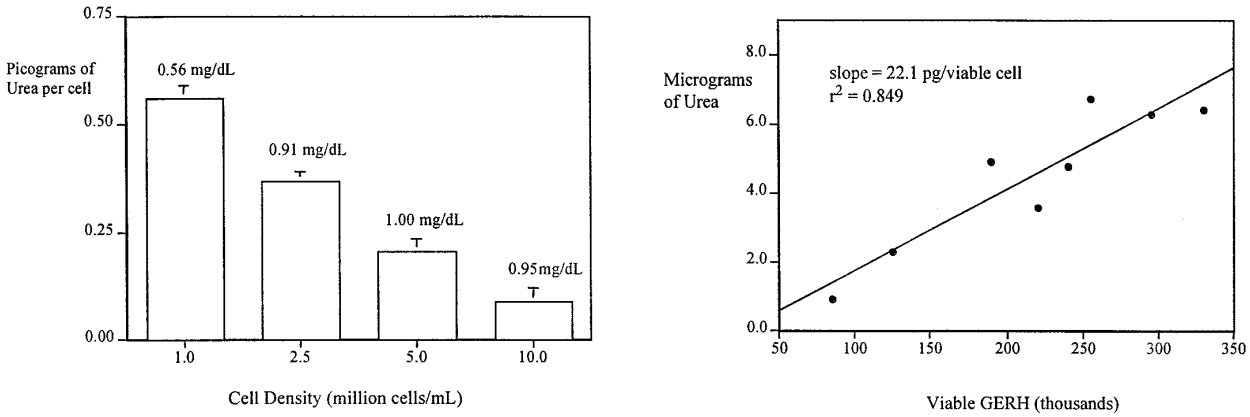


**Fig 2. Left panel,** Urea production (calculated on a per total cell basis) by GERH. Bars are average urea production of 8 gels per group ± standard deviation of mean. **Right panel,** Urea production by GERH on the basis of viable hepatocyte number. Figure suggests that urea production is dependent on viable hepatocytes and not directly proportional to total hepatocyte number. Measures that improve hepatocyte viability and provide greatest number of viable hepatocytes would be expected to provide the greatest metabolic activity.

Most gels were maintained at 37°C under standard conditions (95% humidified air: 5% $CO_2$ incubator). Where specified, other gels were kept anoxic (< 1.5 mm Hg) for 4 to 20 hours before transfer to standard incubator conditions. Anoxia was maintained with a large anaerobic chamber (model 1025; Forma Scientifica Inc, Marietta, Ohio) kept at 37°C.[17] Samples of culture medium and gels containing GERH were collected at specified timepoints after entrapment. Media samples were stored at -20°C before analysis. Some gels were analyzed at the time of sampling (vital staining). Other gels were fixed in formalin or Trump's solution for analysis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay or electron microscopy, respectively (see below).

**Determination of cell viability**

*Quantitation of cell death.* Dead hepatocytes were quantified with a digitized, video archival, epifluorescence microscopy system after vital staining with fluorescein diacetate:ethidium bromide (FDA:EB).[18,19] Dead cells, on the basis of their permeability to EB, were stained orange by this technique; viable cells, capable of converting the non-fluorescent FDA into fluorescein, were stained green. Both apoptotic and necrotic cells were stained orange with this technique. Stock solutions of FDA (5 mg/mL in acetone) and EB (10 μg/mL in phosphate buffered saline) were stored at 4°C. The working FDA:EB (5 μg/mL:10 μg/mL) solution was prepared fresh before each use by adding 10 μL of FDA stock solution to 10 mL of EB stock solution. GERH were incubated in FDA:EB stain for 5 minutes at 37°C and then washed twice in phosphate buffer solution. Viability of hepatocytes (100-200 cells in 3 fields at 400 × magnification) was quantified with Image Pro Plus 3.0 software (Media Cybernetics, Silver Spring, MD) and an epifluorescent microscope (Axiovert; Carl Zeiss, Inc, Thornwood, NY) configured for fluorescein isothiocyanate (450-490 nm excitation filter, 510 nm barrier filter). Cells with green staining cytoplasm are scored as viable. Cells with orange staining nuclei were scored as dead. The percent of viability was determined as: (viable cells/total cells) × 100%.

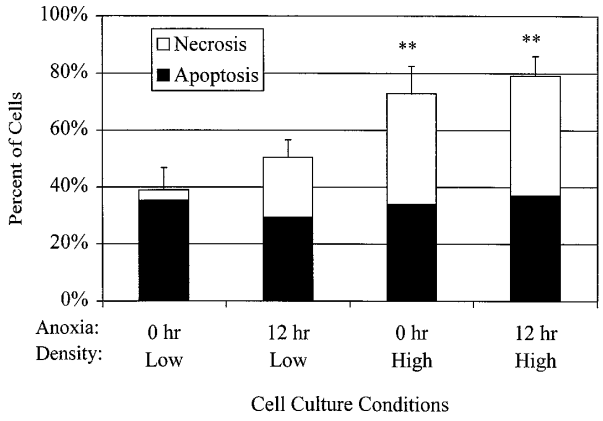


**Fig 3.** Influence of cell density and anoxia on death of GERH after 24 hours in culture. Percentage of cells undergoing apoptosis remained relatively constant, while percent of necrotic cells increased with cell density and 12 hours of anoxic exposure. *Two asterisks, P* value < .05 compared with baseline conditions (0 hours anoxia, low cell density). *Error bar,* Standard deviation of 6 gels/group.

*Quantitation of apoptotic cell death.* A well-established technique, TUNEL assay, was used to identify apoptotic cells.[20,21] Briefly, gels containing GERH were formalin-fixed and embedded in paraf-

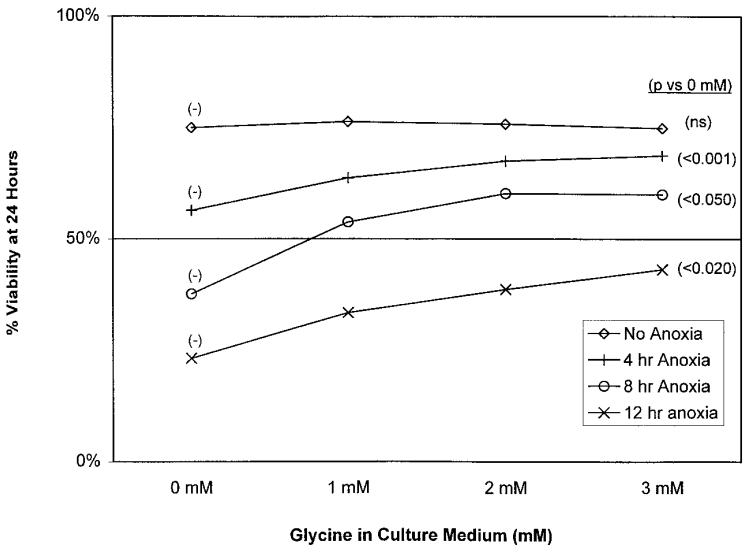


**Fig 4.** The cytoprotective influence of glycine during anoxic injury of GERH is illustrated. Dose response curves shown for low-density cultures of GERH after anoxic (0, 4, 8, and 12 hour) exposure. Viability determined by FDA:EB staining 24 hours after hepatocyte isolation. Values in parentheses represent *P* value of 3 mmol/L versus 0 mmol/L glycine (n = 6 gels/group).

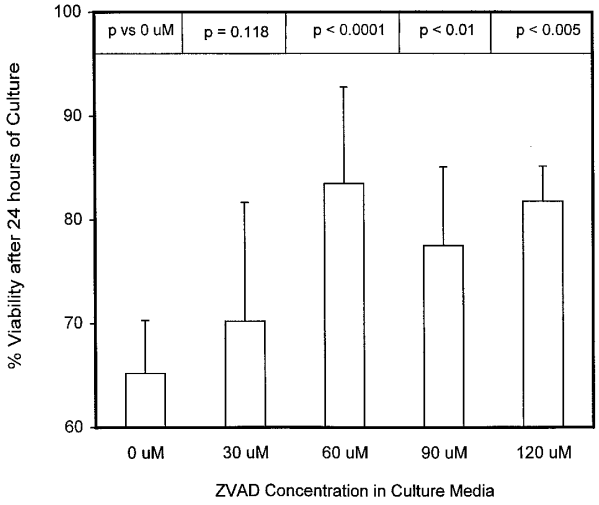


**Fig 5.** The cytoprotective influence of increasing concentrations of ZVAD-fmk during culture of GERH is illustrated. Viability was determined by FDA:EB staining 24 hours after hepatocyte isolation. Values in parentheses represent *P* value compared with baseline (0 mmol/L ZVAD-fmk). *Error bar,* Standard deviation of 6 gels/group.

fin. Thin (5 μm) sections of tissue were transferred to glass slides. Tissue sections were de-waxed and stained with use of a commercially available in situ apoptosis detection kit (Cat #57111-KIT; Oncor, Gaithersburg, MD) based on TUNEL methodology. As directed, residues of digoxigenin-nucleotide were catalytically added to 3'-OH ends of fragmented DNA by means of terminal deoxynucleotidyl transferase (TdT enzyme). A fluorescein-labeled antibody directed against the new residues of digoxigenin-nucleotide was used to stain apoptotic nuclei. Tissue samples were analyzed by epifluorescence microcopy (Axiscope, Carl Zeiss, Inc) configured for FITC. TUNEL-positive cells were evident by means of green staining of the nuclei. Nuclei of nonapoptotic cells were counterstained orange with propidium iodide. The percent of apoptosis was determined as (apoptotic cells/total cells) × 100%.

*Electron microscopy.* Individual gels containing GERH were fixed in Trump's fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.2).[22] Fixed gels were then rinsed for 30 minutes in 3 changes of 0.1 mol/L phosphate buffer, pH 7.2, followed by a 1-hour postfix in phosphate-buffered 1% $OsO_4$. After rinsing in 3 changes of distilled water for 30 minutes, the tissue was stained en bloc with 2% uranyl acetate for 30 minutes at 60°C. The GERH were then rinsed in 3 changes of distilled water, dehydrated in progressive concentrations of ethanol (60-100%) and 100% propylene oxide and embedded in Spur's resin.[23] Thin (90 nm) sections were placed on 200-nm mesh copper grids and stained with lead citrate. Micrographs were taken with an electron microscope (JEOL 1200 EXII;JEOL Institute, Peabody, Mass) operating at 60 kV.

**Urea measurement.** The concentration of urea in the culture media of the GERH was determined by means of a commercially available detection kit (Sigma Diagnostics, St Louis, Mo). Samples of media

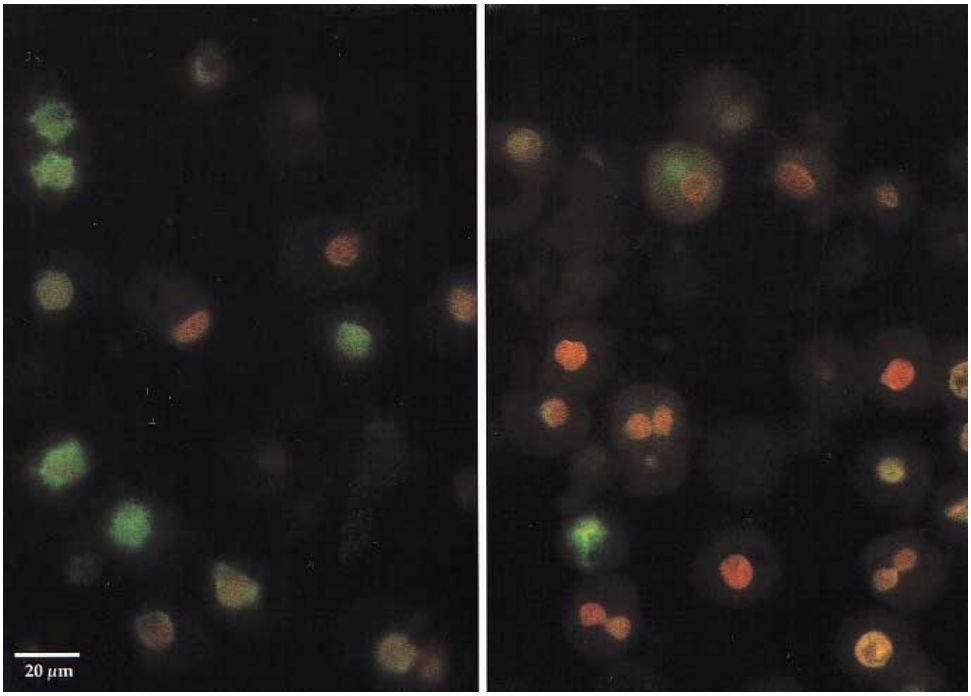


**Fig 6. Left panel,** Apoptotic death of GERH detected in all cultures of GERH by 24 hours after hepatocyte isolation. Green-stained nuclei are apoptotic. Orange-stained nuclei lack DNA fragmentation, which is characteristic of apoptosis. **Right panel,** Fewer cells became apoptotic when the culture media was supplemented with 60 μM ZVAD-fmk. An unusual phenomenon is illustrated in the upper portion of the right figure. Namely, a binucleated rat hepatocyte with 1 apoptotic nucleus and a second normal nucleus suggests anti-apoptotic influence of ZVAD-fmk. Other normal-appearing binucleated cells are observed in this field.

(100 μL) were analyzed with a spectrophotometer (570 nm) as specified in the kit instructions.

**Statistical analysis.** Gels were cultured and studied in groups of 6 to 12 on the basis of the following variables: cell density, anoxic duration, and cytoprotective intervention (none, glycine alone, ZVAD-fmk alone, or glycine and ZVAD-fmk). Endpoints included percent viability, percent apoptosis, and urea production during 24 hours of culture. Experiments were repeated in triplicate. Data were compared between groups by an unpaired 2-sided Student *t* test. Data were considered significant at *P* less than 0.05 and was reported as mean ± standard deviation, unless otherwise indicated.

## RESULTS

**Influence of cell density on viability and function of GERH.** The viability of GERH was measured with FDA:EB staining. This technique identifies necrotic and apoptotic cells by orange nuclear staining and viable cells by green cytoplasmic staining. The number of viable cells declined gradually from harvest (90% ± 1%) during the initial 12 hours after hepatocyte isolation in the absence of glycine and ZVAD-fmk. The rate and extent of decline varied inversely to cell density in all cases. At intermediate ($2.5\text{-}5.0 \times 10^6$ cell/mL) cell density, hepatocyte viability declined during the initial 12 hours and then stabilized above 60% (Fig 1). A similar pattern was observed at low ($1.0 \times 10^6$ cell/mL) cell density with stabilization between 65% and 75% viability as determined by FDA:EB staining. High ($10.0 \times 10^6$ cell/mL) density culture of GERH was associated with the lowest viability (27% ± 10%, $P < .001$ vs $1.0 \times 10^6$ cell/mL) at 24 hours.

Metabolic function of GERH was determined from the concentration of urea in the culture media after 24 hours. Urea concentration (mean ± standard deviation of the mean [SEM]) was determined from 8 gels per group. Experiments were performed in duplicate to confirm the results. Urea concentration at 24 hours was greatest in the intermediate cell density group. When calculated on a per total cell basis (Fig 2, *left panel*), urea production was greatest at low ($1.0 \times 10^6$ cell/mL) cell density. The lowest urea concentrations and lowest production of urea (on a per total cell basis) were observed in the high cell density group, consistent with poor cell viability at high cell density. Urea

**Table I.** Summary of cell death data

| *Culture conditions* | | | *Intermediate cell density* | | *High cell density* | |
|---|---|---|---|---|---|---|
| *Anoxia* | *Glycine (mmol/L)* | *ZVAD-fmk (μmol/L)* | *% Apoptosis* | *% Necrosis* | *% Apoptosis* | *% Necrosis* |
| 0 hours | 0 | 0 | 35 | 4 | 34 | 39 |
| | 3 | 0 | 36 | 2 | 39 | 20(< .05) |
| | 0 | 60 | 23(< .001) | 3 | 16(< .001) | 45 |
| | 3 | 60 | 22(< .005) | 5 | 23(< .05) | 29 |
| 12 hours | 0 | 0 | 29 | 21 | 38 | 42 |
| | 3 | 0 | 28 | 11(< .05) | 39 | 14(< .01) |
| | 0 | 60 | 15(< .01) | 24 | 20(< .05) | 32 |
| | 3 | 60 | 17(< .05) | 13 | 28 | 19(< .05) |

Values in parentheses represent *P* value versus baseline conditions (0 mmol/L glycine, 60 μmol/L ZVAD-fmk, same anoxia, same cell density).
%Necrosis determined from the equation: %necrosis + %apoptosis + %viability = 100%.
%Apoptosis determined by TUNEL assay technique.
%Viability determined by FDA:EB vital stain technique.

**Table II.** Summary of urea production data

| *Culture conditions* | | | *Urea concentration (mg/dL)* | |
|---|---|---|---|---|
| *Anoxia* | *Glycine (mmol/L)* | *ZVAD-fmk (μmol/L)* | *Intermediate cell density* | *High cell density* |
| 0 hours | 0 | 0 | 4.8 ± 1.0 | 5.8 ± 0.9 |
| | 3 | 0 | 5.4 ± 0.8 | 6.3 ± 0.5 |
| | 0 | 60 | 5.5 ± 0.3 | 6.2 ± 0.6 |
| | 3 | 60 | 6.3 ± 1.0(<.05) | 7.8 ± 0.8(< .005) |
| 12 hours | 0 | 0 | 2.0 ± 0.4 | 2.9 ± 0.3 |
| | 3 | 0 | 2.4 ± 0.4 | 3.0 ± 0.6 |
| | 0 | 60 | 2.2 ± 0.4 | 3.3 ± 0.6 |
| | 3 | 60 | 2.9 ± 0.4(< .005) | 3.9 ± 0.2 (< .001) |

Values in parentheses represent *P* value versus baseline (0 mmol/L glycine, 60 μmol/L ZVAD-fmk, same anoxia, same cell density).

concentration was compared to the number of viable cells present in the culture at 24 hours. A direct correlation was observed between the number of viable cells and the production rate of urea (22.1 pg/viable cell, $r^2 = 0.849$) demonstrated in Fig 2 *(right panel)*. These results underscored the importance of cell viability to metabolic performance of a bioartificial liver and the need for cytoprotective measures to maintain or improve hepatocyte viability. Since intermediate cell density provided the greatest number of viable GERH for ureagenesis, intermediate cell density was used for comparison in most of the following experiments.

**Anoxia death and GERH.** The possibility of substrate limitation as a cause of cell death was suggested by the observations that cell viability and ureagenesis both diminished at high cell density. In order to test this hypothesis, intermediate and high cell density cultures of GERH were compared under anoxic (12 hours) and normoxic conditions. Total percent cell death (FDA:EB staining), percent apoptosis (TUNEL assay), and percent necrosis (see Table I for equation) were determined in all 4 groups 24 hours after hepatocyte isolation. Percent apoptosis remained stable at 30%-35% in all 4 groups (Fig 3). Evidence for apoptosis by GERH included the presence of apoptotic bodies and other characteristic changes observed with electron microscopy. The stable percentage of apoptotic GERHs under anoxic and normal culture conditions suggested that apoptosis was triggered by an earlier event, possibly during hepatocyte isolation. Necrosis accounted for the significant increase in cell death at high cell density and after anoxic exposure. The adverse effects of anoxia and high cell density on cell death appeared additive. This observation suggested that oxygen is a potential substrate limiting cell viability at high cell density.

**Effect of glycine during anoxia.** In order to determine the cytoprotective effects of glycine—an agent thought to reduce necrotic death during anoxia—GERH were cultured in media supplemented with glycine. A dose response experiment was first performed by varying the concentration of supplemented glycine (0-3 mmol/L) and the duration of anoxic exposure (0-12 hours) (Fig 4). A concentration-dependent improvement in the via-

bility of GERHs resulted from the addition of glycine to the culture media after 4, 8, and 12 hours of anoxia. The beneficial effect of glycine appeared to reach a plateau at a media concentration of 3 mmol/L glycine. Electron microscopic evidence suggested that improved cell viability in the glycine-treated groups resulted from a reduction in necrotic cell death. There was no beneficial effect of glycine observed under normoxic conditions in this dose-response experiment.

**Effect of ZVAD-fmk.** The anti-apoptotic effects of ZVAD-fmk were first tested on GERH at intermediate cell density and in the absence of anoxia. A dose response experiment was performed with culture media supplemented with a range (0-120 μmol/L) of ZVAD-fmk (Fig 5). Apoptosis was measured with TUNEL assay and confirmed by electron microscopy; total viability was determined by FDA:EB staining. This experiment demonstrated a reduction in apoptosis by GERH's cultured in media supplemented with ZVAD-fmk (Fig 6). A dose-dependent response was observed from 0 to 60 mol/L ZVAD-fmk. Urea production by GERH was also improved in the presence of 60 μmol/L ZVAD-fmk. No additional benefit in viability or urea production was observed above 60 mol/L. In fact, viability declined slightly when GERH were cultured in media supplemented with 120 μmol/L ZVAD-fmk. This was possibly related to a toxic effect of the DMSO solvent used as the vehicle for ZVAD-fmk.

**Effect of ZVAD-fmk and glycine.** Our final experiment was designed to determine if the beneficial effects of glycine and ZVAD-fmk were additive in cultures of GERH. Intermediate and high cell density cultures of GERH were compared during 24 hours of culture, which included normoxic or anoxic (12 hours) exposure. Optimal concentrations of glycine (3 mmol/L) and ZVAD-fmk (60 μmol/L), based on the results of earlier experiments, were used for this final experiment. Endpoints (percent apoptosis, percent necrosis, percent viability, and ureagenesis rate) were determined for each group of 6 to 12 gels. As summarized in Table I, percent apoptosis was reduced by the addition of 60 μmol/L ZVAD-fmk under all 4 conditions (combinations of anoxia and cell density). Percent necrosis was reduced in the presence of 3 mmol/L glycine under both high density culture conditions and at intermediate cell density after exposure to 12 hours of anoxia. The lowest total cell death (percent apoptosis and percent necrosis) was observed if GERH were maintained in media supplemented with both 3mmol/L glycine and 60 μmol/L ZVAD-fmk (Table I). Urea production of GERH was also greatest in media supplemented with both cytoprotective agents (Table II). Individual agents showed a trend towards improved function, though both glycine and ZVAD-fmk were necessary to achieve significance with 6 gels per group. These data confirm that the cytoprotective effects of glycine and ZVAD-fmk can be additive. In addition, the data indicates that glycine reduces necrotic cell death, while ZVAD-fmk improves cell viability and function through reduced apoptosis of GERHs.

## DISCUSSION

Multiple configurations, including gel entrapment, have been proposed for hepatocyte culture in a BAL. Along with the biologic benefits of gel entrapment (providing a basement membrane matrix, allowing cell-cell contact between cultured hepatocytes and providing a porous latticework for mass transport of substrate and waste materials), an accurate determination of percent viability, percent apoptosis and percent necrosis was possible in our experiments because of gel entrapment. In contrast to surface culture of epithelial cells in which dead, nonadherent hepatocytes are lost in the culture medium, dead cells remain entrapped within the 3-dimensional collagen latticework after gel-entrapment. The number of nonadherent, free-floating cells was negligible in these experiments.

We observed that approximately 30% of GERH underwent apoptotic death by 24 hours after hepatocyte isolation regardless of cell density or anoxic conditions. In comparison, necrosis of GERH was a minor problem at low cell density and became a major cause of cell death at high density and under anoxic conditions. Along with confirmation of the significance of necrotic and apoptotic cell death, these observations raised the possibility that viability of GERH could be improved by treatment with anti-necrotic and anti-apoptotic agents such as glycine and ZVAD-fmk, respectively. In fact, the current set of experiments demonstrated a cytoprotective influence of ZVAD-fmk and glycine when supplemented to the culture medium of hepatocytes in a gel-entrapment configuration. The anti-apoptotic activity of ZVAD-fmk, a non-specific caspase inhibitor, was dependent on its concentration in the culture medium with 60 μmol/L ZVAD-fmk appearing to be the optimum dose. The greatest anti-necrosis effect was observed after supplementation of William's E medium (0.6 mmol/L glycine from manufacturer) with an additional concentration of 3 mmol/L glycine.

The beneficial effects of glycine as an anti-necrosis agent during anoxic conditions are well recognized.[13-15,17] In the current set of experiments,

glycine did not appear to be beneficial under low-density culture conditions ($<5 \times 10^6$ cells per mL of gel). However, at high cell density culture, beneficial effects of glycine were identified. These results suggest a potential therapeutic benefit of glycine treatment of hepatocytes cultured at high density in a bioartificial liver. Of note, neurotoxicity has been observed in patients receiving glycine bladder irrigation after transurethral-prostatectomy.[24] Nausea, vomiting, and transient neurologic events developed in some patients if serum glycine concentrations rose above 5 mmol/L. No neurologic side effects were encountered with serum concentrations of glycine below 3 mmol/L, the concentration associated with optimal cytoprotection in our study. Therefore, neurologic complications would be unlikely if 3 mmol/L glycine were present in the culture medium before clinical use of the BAL. Physiologic concentration of glycine in the peripheral blood of the fasted rat is 0.4 mmol/L for comparison.

The assumption that more hepatocytes ensure greater metabolic performance of a hepatocyte BAL must be reconsidered with the understanding that tissue hypoxia and necrotic cell death are more likely at high cell density. Optimal performance of a hepatocyte BAL involves culture conditions, which maximize the number of viable hepatocytes within the device. Of note, even in the setting of glycine-supplemented media, high cell density cultures of GERH provided less ureagenesis than intermediate cell density cultures under otherwise similar culture conditions (Table II). At a minimum, our studies highlight the problems that must be considered when hepatocytes are cultivated at high cell density. It is possible that higher cell densities can be used within a BAL under perfused conditions such as hemoperfusion during extracorporeal therapy. Mass transfer is improved significantly by convective flow in a perfused BAL device when compared with simple diffusion under static culture conditions.[25] Also, oxygen solubility of culture media is significantly less than oxygen-carrying capacity of blood. Whole blood perfusion of a BAL, therefore, may have a beneficial effect on hepatocyte viability and function by improved oxygen delivery and reduced necrotic cell death within the BAL.

Our data is consistent with the notion that apoptosis results during epithelial cell isolation and that apoptosis may be problematic in other BAL designs that use isolated hepatocytes. The specific cause of apoptosis during hepatocyte isolation is open to speculation. It is known that apoptosis is mediated by intracellular proteases, such as caspases[26,27]. Several modes of caspase activation have been identified,[28] including the loss of epithelial cell-matrix (receptor-ligand) interaction during cell isolation.[29,30] According to this hypothesis, integrin receptors on anchorage-dependent epithelial cells such as Chinese hamster ovary cells ($\alpha5\beta1$ integrin)[31] and hepatocytes ($\alpha1\beta1$ integrin, $\alpha5\beta1$, $\alpha9\beta1$ integrins)[32] normally bind to extracellular matrix components. These matrix components supply ligands that appear to be necessary to maintain cell viability. [33, 34] It is also possible that inappropriate matrix receptor interactions can trigger apoptosis.[35,36] The process of apoptosis resulting from the loss of normal integrin-ligand interaction is known as anoikis or "homelessness."[37,38] Anoikis probably serves a normal role in the death of epithelial cells separated from their basement membrane and may even prevent metastatic spread of some epithelial tumors. Apoptosis is undesirable, however, after isolation of hepatocytes intended for use in a BAL.

The current study supports the use of caspase inhibition for increased viability and improved metabolic function under conditions prone to apoptosis, such as after hepatocyte isolation. In the current experiments, the caspase inhibitor (ZVAD-fmk) was supplemented to culture media of GERH. It is possible that further improvement in hepatocyte viability (reduced apoptosis) could result from pretreatment of the donor animal with a caspase inhibitor prior to hepatocyte isolation. The presence of caspase inhibitors at the time of collagenase infusion and hepatocyte isolation might further reduce the caspase cascade believed to result from the interruption of natural receptor ligand interactions.[34] In addition, pretreatment with a caspase inhibitor, such as ZVAD-fmk, may improve the viability of cryopreserved (isolated) hepatocytes. Cryopreservation has been shown to increase apoptotic death in other epithelial cell types, such as isolated human corneal keratocytes.[39] Reducing cell death associated with cryopreservation is of importance since cryopreserved hepatocytes are used in at least one clinical BAL system.[4]

In summary, our data suggest that minimizing the injury from hepatocyte isolation (ie, caspase activation) and reducing the ischemic injury inherent with high-density cell culture should improve viability and function of isolated hepatocytes. In particular, cytoprotective agents such as ZVAD-fmk and glycine may have a beneficial influence on the performance of a bioartificial organ, such as the hepatocyte BAL. Other applications of isolated hepatocyte culture may also benefit from these cytoprotective agents.

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