Small human hepatocytes for treatment of alcohol addicts? Metabolism of ethanol, diazepam and oxazepam in rotary cell culture*

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* Original publication: M Pavlic, K Libiseller, M Hermann, P Hengster, R Margreiter, M Wurm (2007) Small human hepatocytes in rotary culture for treatment of alcohol addicts? A pilot study. Alcohol Clin Exp Res, Vol 31, No 5: 729-36.

Abstract

Objective: Alcohol addict patients may be treated with benzodiazepines to prevent withdrawal symptoms. If facing liver failure, current approaches to bridge them to liver transplantation include culturing human cells to take over basic metabolic functions for a certain time.

Material and Methods: The potential of small human hepatocytes (SH), grown in a rotary cell culture system, to metabolize alcohol and the benzodiazepines oxazepam and diazepam was evaluated. Cell supernatants were analysed using HS-GC-FID (ethanol) and GC-ECD (benzodiazepines). Control experiments were performed with SV40-immortalized HEP cells and cell respective drug free media. Cell viability was supervised with confocal microscopy.

Results and Discussion: The experiments show that SH in rotary culture are obviously able to metabolize ethanol in reasonable amounts compared to evaporation controls ($p < 0.01$). In addition, SH reduce diazepam and oxazepam which indicates the presence of functional cytochrome P450 enzymes and the ability of SH to perform conjugation. Moreover, basic metabolic cell activities such as glucose consumption, albumin and urea production were analysed. These parameters were not significantly influenced by the drugs used, which is a precondition for clinical use of these cells. Nevertheless, LDH release was significantly increased in SH incubated with either ethanol ($p < 0.05$) or diazepam ($p < 0.005$), which indicates enhanced cell death in these cultures. Stable viability at or above 90%, however, suggests that cell proliferation in rotary culture is able to keep up with drug-induced cell death. This preliminary study therefore shows that SH in rotary cell culture are basically suited to bridge alcohol-abusing and/or benzodiazepine-treated patients undergoing liver failure.

1. Introduction

Alcohol abuse is the main contributory factor to 50% of deaths from liver cirrhosis, but normally does not cause peracute liver failure [1]. Therapeutic strategies at a means of preventing final liver failure comprise abstinence of further alcohol consumption [2]. These patients need special care to prevent withdrawal symptoms that may be life-threatening [3], which is commonly done by a long-acting benzodiazepine, e.g. diazepam [4]. For sudden symptoms of withdrawal such as tremor, sweating, tachycardia or agitation, the benzodiazepine oxazepam has been established [5].

In spite of these strategies, liver failure can not be prevented in every case. For such patients orthotopic liver transplantation (OLT) is still the only form of final treatment [6]. Donor shortage does not allow every patient to benefit from liver transplantation. Therefore a host of liver support systems for bridging patients to transplantation have been developed, among them bioartificial liver (BAL) support devices that use a reactor filled with hepatocytes. Discussion about the best type of device and cell choice for BAL is still ongoing [7]. It is obvious that human hepatocytes should be the primary choice, as for example porcine endogenous retrovirus (PERV) is considered to be one of the major risks in the application of xenogenic cells [8].

Recently, small human hepatocytes (SH) were introduced as a possible alternative for liver repopulation [9], being hepatic progenitor cells. For rat small hepatocytes it was shown that they express liver cell functions [10], but very little is known about their potential to metabolize xenobiotics.

One aim of the present study was to evaluate whether SH, grown in a prototype BAL using a rotating cell culture system (RCCS), have the potential to metabolize regarding ethanol and the benzodiazepines diazepam and oxazepam. These drugs can understandably be present in patients facing liver failure. In addition, glucose consumption, albumin secretion, release of LDH and urea synthesis were determined to examine potentially damaging effects of xenobiotics on the cells.

2. Material and Methods

Standard cell culture

Cells were seeded in six-well plates (Greiner Bio-one, Frieckenhausen, Germany). Small human hepatocytes (SH) were were isolated from human liver by differential centrifugation by Dr. Stefano Buttiglieri (University of Turin, Italy). SH were cultivated in MDBK-MM (Sigma, St. Louis, MO) medium supplemented with fetal calf serum (FCS) (JRH Bioscience, Lenexa, Kansas), glutamine, penicillin/streptomycin (P/S) (both PAA, Pasching, Austria) and insulin-transferrin-selenite (ITS) (Sigma, St. Louis, MO). HEP cells (courtesy of Dr. Jan Hengstler, University of Leipzig, Germany), i.e. SV40-immortalized human hepatocytes, were used as control and were grown in RPMI 1640 media (PAA, Pasching, Austria) supplemented with FCS, glutamine and P/S.

The BAL prototype

The BAL prototype is a constantly perfused RCCS currently being developed. A chamber (85 ml) containing the cells is placed in the rotation unit and linked to a media circuit by bundles of porous albuflow® fibers (Fresenius Medical Care, Bad Homburg, Germany) passing the chamber and permitting mass transfer by diffusion. An oxygenation device is attached for oxygen, nitrogen and CO_2. A media supply circuit is driven by a peristaltic pump. SH cells were attached to cytodex 3 beads (GE Healthcare, Chalfont St. Giles, UK) and then grown in rotating culture.

Sampling

Cells were cultured for two days (standard) or up to twelve days (rotating cell culture). Samples of medium were collected after 0, 6, 12, 24 and 48 hours following administration of xenobiotics (ethanol, oxazepam, diazepam) or for ethanol-evaporation control experiments. SH were collected after 0, 1, 5, 8 and 12 days of culture to determine activity and cell count.

Determination of ethanol

Ethanol (p.a. grade, Merck, Darmstadt, Germany) was added to a final concentration of 0.75 g/l. Ethanol concentrations were determined using headspace gas chromatography with flame ionization detection (HS GC-FID) and t-butanol as internal standard. The headspace injection volume was split into two different columns (PE-BAC1 [30 m x 0.32 mm x 1.8 µm] and PE-BAC2 [30 m x 0.32 mm x1.2 µm], Perkin Elmer, Vienna). Headspace conditions: thermostating 20 min at 60°C, needle and transfer temperature 90°C, pressurization time: 1 min, injection temperature 100°C. GC conditions: 3 min 35°C, FID: 240°C. The average value was calculated from the two thus obtained results.

Determination of benzodiazepines

Diazepam and oxazepam (1 µg/ml, Pharmacy of Innsbruck Medical University Hospital, Austria) were added to the culture. Analyses of diazepam and oxazepam were performed using gas chromatography combined with electron capture detection (GC-ECD) on an Autosystem XL device (Perkin Elmer, Vienna, Austria) using flurazepam (1 mg/ml in methanol) as internal standard. Solid-phase extraction was performed using SPE-ED Scan ABN columns (Applied Separations, Allentown, PA) that were conditioned with methanol and phosphate buffer, pH 6.0. After rinsing the samples, columns were washed with distilled water, acetic acid and a methanol water solution, centrifuged and dried under nitrogen. Elution was performed with an anhydrous solvent mixture of dichloromethane, i-propanol and ammonia. The eluate was evaporated under nitrogen and reconstituted in ethylacetate. A HP-5 MS column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; J&W Scientific, Folsom, CA) was used. Operation conditions were as follows: carrier gas hydrogen; injection volume 1 µl, injection temperature 250°C; oven: 100°C, 20°C/min to 220°C, hold for 15 min; 20°C/min to 300°C, hold for 5 min; ECD temperature 350°C.

Standard cell culture parameters

The cell culture parameters glucose consumption, lactate dehydrogenase (LDH) release, albumin secretion and urea production (after addition of NH4Cl) were monitored alone and in the presence of ethanol and benzodiazepines using commercial kits according to the manufacturer's instructions (Roche Pharma, Reinach, Switzerland, for glucose, LDH and urea; ZLB Behring, Vienna, Austria, for albumine).

Confocal microscopy

A microlens-enhanced Nipkow disk-based UltraVIEW RS confocal scanner (Perkin Elmer, Wellesey, MA) mounted on an Olympus IX-70 inverse microscope (Olympus, Nagano, Japan) was used. Cell morphology was visualized via staining of membrane glycoproteins with fluorescein-labeled wheat germ agglutinin (WGA, Molecular Probes, Eugene, OR). WGA also binds to the cytodex beads. To analyze mitochondrial inner membrane potential, cells were stained with tetramethylrhodamine methyl ester (TMRM, Sigma, St. Louis, MO). Cells were sequentially excited at 488 nm (WGA) and 567 nm (TMRM). For determination of cell count and viability, three representative visual regions of interest (ROI) were counted.

Statistical analysis

All descriptive statistical parameters were generated with MS Excel 2002. Performed Student's T - test was double-sided. All data are given as mean value ± standard error, if not otherwise indicated. Results were considered statistically significant at a probability of 95% ($p < 0.05$).

3. Results

Ethanol

SH and HEP were incubated with 0.75 g/l ethanol under standard cell culture conditions. Determination of ethanol over a period of 24 h revealed no difference in ethanol reduction with HEP cells and in evaporation control experiments without cells. In contrast, ethanol was reduced by 2.1 mg in wells with SH cells as compared to 1.8 mg in wells without cells. If ethanol evaporation is considered, 0.06 ± 0.01 mg ethanol per 10^6 SHs was metabolized.

Further experiments were performed using the prototype BAL charged with SH. Measurements revealed a mean ethanol reduction of on average 48 ± 11% after 24 h and 59 ± 9% after 48 h and showed a statistically significant difference compared to evaporation control (21 ± 5% after 24 h, $p < 0.05$, and by 27 ± 5% after 48 h, $p < 0.01$, *Figure 1*).

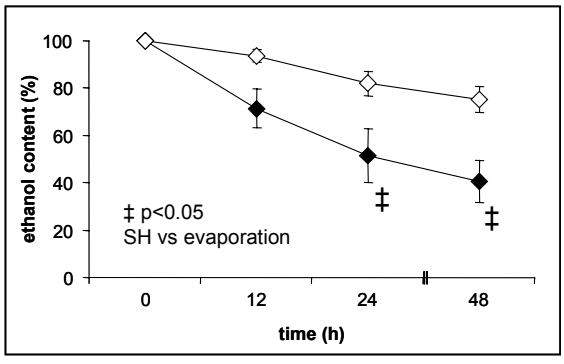

Fig. 1: Metabolism and evaporation of ethanol in RCCS. Reduction in ethanol in RCCS by SH (filled symbols) is significantly higher than evaporation (open symbols).

SH metabolized 0.10 ± 0.03 mg ethanol per 10^6 cells in the course of 24 h, equivalent to 4 ± 1.2 µg per 10^6 cells per hour. These results indicate that SH in RCCS are able to metabolize ethanol nearly twice as fast as in standard cell culture.

Benzodiazepines

In standard culture, SH showed a 36% decrease from on average 990 ± 180 µg/l oxazepam to 635 ± 55 µg/l after 48 h. However, as the results in 24 h did not show a significant decrease, the reduction ability must therefore be seen as a trend only.

Further experiments were performed with SH cultivated in RCCS. Oxazepam was reduced by SH to 36 ± 6% within the first 24 h and to 33 ± 7% after 48 h of culture. After 24 h diazepam was reduced to 30 ± 4% and after 48 h to 19 ± 5%. *Figure 2* depicts the mean reduction in diazepam and oxazepam by SH cells. Apparently elimination follows a first-order kinetic. Analysis of cell culture medium without SH to which both benzodiazepines were added revealed the originally spiked concentration after storage of 48 h.

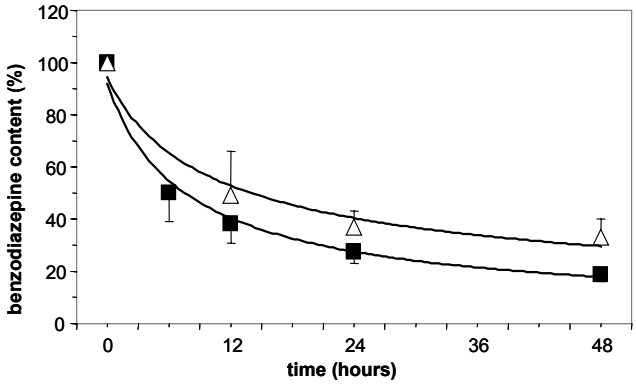

Fig. 2: Elimination curves of diazepam (filled symbols, r2 = 0.9875) and oxazepam (open symbols, r2 = 0.963) by SH in RCCS.

Metabolic activities and cell viability

In standard culture, glucose consumption, LDH release, albumin and urea production of SH and HEP cells alone and in the presence of ethanol and oxazepam were monitored over 48 hours. Neither parameter was significantly altered by any of the added xenobiotics or stayed below detection limits (data not shown).

Table 1 shows the metabolic parameters glucose consumption, LDH release, albumin and urea production per 10^6 SH cells and 24 h, cultivated alone (control) as well as in the presence of ethanol, diazepam or oxazepam in RCCS.

Table 1: Basic metabolic activities of SH in RCCS. Glucose consumption, urea production and albumin secretion (mg/24h/10^6 SH); LDH production (mU/24h/10^6 SH); ‡ $p < 0.05$, § $p < 0.005$, * $p = 0.08$ vs drug free controls.

	Glucose	Urea	LDH	Albumin
Control	0.90 ± 0.18	0.05 ± 0.016	9 ± 0.9	0.018 ± 0.011
Ethanol	0.90 ± 0.12	0.04 ± 0.005	56 ± 8.0 ‡	0.005 ± 0.001
Diazepam	1.30 ± 0.21	0.04 ± 0.005	77 ± 13 §	0.006 ± 0.001
Oxazepam	0.94 ± 0.11	0.04 ± 0.006	40 ± 16	0.002 ± 0.001*

Glucose consumption of SH in RCCS was not altered significantly by addition of xenobiotics. When diazepam was added, glucose consumption of SH cells was slightly, but not significantly enhanced ($p = 0.09$).

Basic urea production was lowered slightly but not significantly by addition of any of the xenobiotics.

On the contrary, LDH release from SH in rotating cell culture was significantly enhanced by addition of some xenobiotics. Addition of ethanol resulted in a six-fold LDH release ($p < 0.05$), whereas diazepam induced even a nine-fold increase ($p < 0.005$). Oxazepam still enhanced LDH release 4.5-fold as compared to the control experiments, but these results were not statistically significant ($p = 0.09$).

Albumin production was reduced by adding xenobiotics, although not significantly. Addition of ethanol resulted in a 34% decrease per 10^6 SH cells and 24 h ($p = 0.13$), whereas diazepam induced a 30% decrease ($p = 0.14$). Oxazepam even reduced albumin production by nine times, but the high variation in values prevented a significant difference from being obtained ($p = 0.08$).

Confocal microscopy showed cell viability to be about 95% in control cell cultures. This value was not altered by the presence of ethanol or oxazepam, whereas addition of diazepam slightly decreased cell viability to approximately 90%.

SH were grown in RCCS for altogether 12 days. Xenobiotics were added after 5 and after 9 days. Cell count in untreated cultures rose from 5 x 10^7 cells to approximately 4 x 10^8 cells on day 12, following a logarithmic trend. Incubation with the tested xenobiotics did not change the logarithmic growth trend but reduced the steepness, resulting in slightly decreased growth rates. Oxazepam reduced cell growth by approximately 6%, diazepam by 15% and ethanol by 18%.

4. Discussion

Treatment of ALD and liver failure is a common medical task. Currently, several approaches are being evaluated to "bridge" patients over the period between liver failure and possible liver transplantation. As these approaches aim to substitute selected metabolic liver functions only, cultured liver cells have been the obvious solution to meet this challenge up to now. Cells for such a liver support must meet at least the following requirements: they must maintain their metabolic capacities under culture conditions, and they must be able to be cultured in amounts that can support an adult liver for a reasonable time.

One aim of this study was to evaluate whether and how human SH can be used for alcohol-addicted patients suffering liver failure and needing withdrawal prophylaxis, which is often accomplished with benzodiazepines.

Preliminary experiments were performed with human SH and SV40-immortalized HEP cells under standard cell culture conditions. Ethanol decrease in HEP cell culture did not differ from evaporation control. This was not unexpected, as immortalized cells dedifferentiate towards a highly proliferative phenotype, which is often accompanied by a loss of specific functions. Furthermore, glucose consumption, urea and LDH production as well as albumin secretion did not differ from drug-free HEP cultures. These preliminary findings show that ethanol seems to act quite inertly in HEP cells.

The second step of our experiments then involved SH growth using a rotating cell culture system. Cells cultured to a mean density of 3.8×10^6 cells per ml were indeed able to metabolize a mean, evaporation-corrected ethanol amount of 4 µg per 10^6cells per hour (*Figure 1*). In comparison, the human body metabolizes about 100 mg ethanol per hour and kg body weight. To reach this metabolism rate, our BAL prototype would have to maintain 2.5×10^{10} cells. The small prototype device used has already achieved cell growth at a density of more than double standard rates. Currently a RCCS for clinical applications is being developed.

Addition of ethanol affected some metabolic duties, but not always significantly: urea production decreased by about 22% and albumin production was reduced by 70%. Consumption of glucose even remained completely unaffected. As ethanol is a well-known energy source, our results suggest that SH cells may be able to gain energy from ethanol metabolism. However, LDH release was about six-fold greater, which is an indicator of increased cell death. Actually, overall cell growth was diminished by about 18% in the presence of ethanol, but viability of cells stayed stable at around 95%, as determined by confocal microscopy. We therefore assume that ongoing regeneration in the rotating culture was able to replace dead cells to a sufficient extent and to maintain satisfactory cell growth. In the presence of ethanol, SH might therefore prove to be a valuable source of BAL support.

Experiments with SH and HEP cells in standard culture spiked with oxazepam revealed that the tested metabolic functions were not affected. In the human liver, oxazepam is not further metabolized, but undergoes conjugation to glucuronic acid and is then eliminated in the urine [11]. However, oxazepam seems to be more or less inert in HEP cells. SH cells, on the contrary, showed a trend to oxazepam being reduced to about 64% after 48 h. SH therefore seem to be able to conjugate.

Finally, diazepam and oxazepam were added to SH in RCCS. Diazepam is metabolized via the cytochrome P450 (CYP) enzyme family in the human liver. CYP catalyze for example oxidation and reduction reactions and act as an oxygenase [12], thus explaining the possibility for complex interactions. Diazepam shows a high affinity to CYP 3A4 [13]. SH were able to reduce more than 80% of the added diazepam within 48 h, Figure 2 shows that the reduction follows a first-order kinetic. The results indicate that SH cells possess enzymes of the CYP family. Evaluation is necessary what kind of CYP enzymes are actually expressed in SH.

Basic metabolic functions were altered in the presence of diazepam, among which the nearly 900% increase in LDH release was statistically significant ($p < 0.005$). Glucose consumption increased by 40%, indicating a higher rate of metabolism. Again, confocal microscopy of samples revealed cells to have sufficient viability about 90%, although slightly below that of controls (95%). Diazepam therefore seems to be a stress factor for SH in RCCS, decreasing growth by on average 15%. The obvious reduction of diazepam indicates the ability of SH to cope with this drug, albeit at high metabolic costs.

SH in RCCS were able to reduce oxazepam by 67% within 48 hours. The elimination curve showed again a first order kinetic. SH seem to be able to conjugate, another basic function of cells planned for liver support systems. Interestingly, comparison of metabolic parameters of SH revealed no significant differences from those of drug-free controls with the exception of albumin secretion. The latter was reduced nearly nine-fold, but was subject to great fluctuations. Obviously, conjugating and protein-producing tasks interact in the cells in the presence of oxazepam. Confocal microscopy proved a nearly unchanged viability, and cell growth was reduced by only about 6%. The quite unspecific task of conjugation is obviously easier to perform by SH than a metabolic pathway using CYP enzymes. Our data indicate that, provided that the medical state of a patient allows such a decision, oxazepam should be the substance of first choice, causing less interaction within SH in RCCS.

5. Conclusion

Small human hepatocytes (SH) cultured in a rotating cell culture system (RCCS) are able to metabolize ethanol in reasonable amounts and to reduce oxazepam and diazepam, which proves their ability to conjugate to glucuronic acid and the presence of functional cytochrome P450 enzymes. Basic metabolic functions of SH are not significantly influenced by the drugs used, which is a precondition for clinical use of these cells, but significantly increased LDH release and reduced growth rates indicate enhanced cell death in presence of ethanol or diazepam. However, stable cell viability suggests that the RCCS is able to keep up with ethanol- and diazepam-induced cell death. If it is allowed from a medical point of view, oxazepam should nevertheless be the benzodiazepine of choice in therapy. Altogether, SH seem to be basically well-suited to support alcohol-abusing and/or benzodiazepine-treated patients undergoing liver failure. Future experiments will address metabolic pathways in detail and the enlargement of the rotating cell culture device. Application of analytical methods developed for forensic toxicological purposes enabled evaluation of cell metabolism abilities, proving again the importance of linking laboratory and clinical medicine.

6. Literature

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