Culture of bovine hepatocytes: a non-perfusion technique for cell isolation

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Abstract In this work we have studied the isolation and culture of mature bovine hepatocytes on plastic dishes without exogenous matrix. The liver has been disaggregated in a collagenase solution instead of undergoing a perfusion step. After a few days in culture, the plates showed several clusters of different cell types. Although the average yield was $1.60 \pm 0.57 \times 10^8$ viable liver cells per gram of tissue, these cultures were formed by non-parenchymal cells and only very few or none by parenchymal cells. In these cultures, actin structures used as a marker for Stellate (Ito) cells have been visualized by immunocytochemical techniques. In order to increase the proportion of parenchymal cells a centrifugation on Percoll, which separates cell sub-populations, has been introduced. Though the

yield was lower than in the previous method, these purified cultures were only composed of hepatocytes. It has been shown that these cells exhibited albumin synthesis, which is a specific hepatocytes function. In addition, these cultures were capable of producing metabolites of 7-ethoxycoumarin at a higher rate than non purified cell cultures. Therefore this simplified procedure for the isolation and culture of functional and viable hepatocytes may be applied for in vitro studies in bovine.

Keywords Bovine hepatocytes · Cell culture · Cell isolation · Non-perfusion · Purification of parenchymal cells · Bovine liver

Abbreviations

BSA	bovine serum albumin
FBS	foetal bovine serum
PBS	phosphate buffered saline
ATB	antibiotics
DAB	3,3' diaminobenzidinetetrachloride chromogenic-substrate system
7-EC	7-ethoxycoumarin
7-OH	7-hydroxycoumarin

Introduction

The liver is an organ responsible for many metabolic, synthetic and storage functions. It is also responsible for the activation and detoxification of endogenous

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and exogenous chemicals. Culturing primary hepatocytes as monolayer primary cultures may provide an efficient system for the study of long term hormonal and nutrient effects on these cells function. However, there are some limitations in these cultures, including non-homogeneity of cell population. Thus, in order to understand better the normal liver function, it is important to separate and culture its different cell types. Parenchymal cells constitute 93% of the total volume of liver cells, though they represent 65% of the relative number of different cell types. Stellate (Ito) cells are a common contamination of the parenchymal cells. They are responsible for 80–90% of the total vitamin A storage and these cells also synthesize several components of the extracellular matrix, such as collagen, laminin, fibronectin and glycosaminoglycans (Berry and Friend 1969; Berry et al. 1991; Alston-Smith and Pertoft 1995). The separation of hepatocytes from that type of cells probably reduces their adhesion ability in non-matrix-coated plastic dishes. On the other hand, Ito cells could be an important competitive factor with parenchymal cells on space and nutrient resources because of their high multiplication rate.

Adult hepatocytes in culture are quiescent, non-dividing cells. Previous reports have indicated that growth regulation of mature hepatocytes is cell density dependent. At low-densities, hepatocytes proliferate, whereas at high densities hepatocytes do not proliferate, but differentiate into liver specific phenotypes and specific markers are induced such as albumin production (Nakamura et al. 1983a, b).

Usually a perfusion step is an integral part of cell disaggregation method (Forsell et al. 1985; Shull et al. 1986; Donkin and Armentano 1993). However, when freshly isolated samples of liver from large mammals, like bovine, are used, the perfusion step is technologically demanding. Another disadvantage is that the liver must be entirely fresh during perfusion; if this is not the case the blood clots may rapidly form within the liver and interfere with the perfusion (Gibson-D'Ambrosio and D'Ambrosio 1996; Fry et al. 1976).

The purpose of this paper is to present a non-perfusion technique for the separation of bovine hepatocytes in culture, and to characterize the cell types obtained with or without purification of parenchymal cells.

Primary hepatocyte cultures are known to conduct phases I and II metabolism (Shull et al. 1986). A demonstration of experimental utilization of the cultures is included to show that these cells are able to metabolize a xenobiotic in the expected products.

Materials

Reagents and solutions

Collagenase type II, 390 units mg^{-1} , Hanks' balanced salt solution (HBSS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (1:1), with L-glutamine and pyridoxine hydrochloride (D-MEM/F12), crystalline bovine insulin (Zinc), bovine albumin fraction V, Fungizone antimycotic (250 μg of amphotericin B and 250 μg of sodium desoxycholate per ml), from Gibco BRL Invitrogen Corporation (GIBCO^{TM}, Invitrogen, Argentina).

Hepes-buffered salt solution (HBSS), pH 7.8, containing 1.78 mM NaHCO_3 , 5.5 mM D-Glucose, 10 mM Hepes, (HBSS-Hepes).

EGTA 10 mM in PBS, pH kept at 7.0 with 10 M NaOH, filtered to sterilized and added to PBS to a final concentration of 0.1 M (PBS-free).

ATB solution: Penicillin G (Na-Salt) 300.000 UI, Gentamycin 0.15 g, Streptomycin 0.20 g, PBS 300 ml. Antibiotics were from Droguería Saporiti, Av. Del Campo 1443, Buenos Aires, Argentina.

Percoll, sterile (Pharmacia).

Protein Block (normal goat serum), Multilink, Avidin were from Biogenex (4600 Norris Canyon Road, San Ramon, CA 94583, USA).

Anti-BSA: Rabbit anti-bovine serum albumin, polyclonal antibody, developed in our laboratory. This antibody had been previously tested in an enzymeimmunoassay, in a dilution (1:1000) from the serum, showing signal in a 1/1000 dilution of serum.

Mouse monoclonal antibody muscle specific ($\alpha\gamma$ -actin) mouse monoclonal antibody was from Novocastra Laboratories Ltd. (Balliol Business Park West, Benton Lane, Newcastle upon Tyne NE12 8EW, United Kingdom). This antibody cross-reacts with bovine muscle actins.

Blocking kit from Vector Laboratories, Inc. (330 Ingold Road, Burlingame, CA 94010 USA).

Foetal bovine serum (FBS) was from Laboratorio Toubey S.R.L. (Pedro de Jeréz 450, Buenos Aires 1427, Argentina).

3,3'-diaminobenzidinetetrachloride (DAB) (0.02%) in Tris-HCl buffer (0.05 M), pH 7.6, and 100 μ l H₂O₂ added just before use.

β -glucuronidase Type H 1, with sulfatase activity, from Hellix Pomatia 486,000 units/g, 7-ethoxycoumarin (7-EC) and 7-hidroxicoumarin (7-OH), were from SIGMA.

Methods

Isolation of hepatocytes

Livers from healthy calves and steers were collected from the slaughterhouse. Immediately after the animal was slaughtered, the complete liver was removed from the body, and the caudate lobe was excised by cutting it across its base. After a brief dip in ethanol 70%, for 30 s, to reduce superficial contamination, the liver was shipped into a sterile bag, in wet ice, to the laboratory. The shipping time was less than 30 min.

Tissue pieces from the centre of the liver sample were aseptically transferred to a flask containing PBS ATB X2, in a sterile area. The tissue was finely minced with scissors and washed several times with PBS-free to remove blood clots. After a last wash with PBS (without EGTA), the sample was transferred to a conical flask with a cold collagenase solution (Collagenase II, 500 ng ml⁻¹ in HBSS-Hepes buffer), and was softly stirred for 12 min (150 ml of Collagenase solution per 1–5 g of tissue). At the end of the incubation 10 ml of FBS and 100 ml of ice cold HBSS containing 5% FBS were added. The cell suspension was filtrated through cheesecloth and centrifuged at 50 g for 5 min at 4°C. The supernatant was discarded and the cell pellet was washed with PBS ATB X2 and centrifuged twice at 150 g for 2 min each. Finally the pellet was suspended either in PBS for further purification of parenchymal cells, or in growth medium to be cultured. Cell viability was determined by Trypan blue exclusion method (Shull et al. 1986).

Purification of parenchymal cells

To obtain a pure subpopulation of parenchymal cells, 5 ml of the cell suspension was layered over 15 ml of Percoll 60% (9 parts of Percoll with 1 part of PBS

X10, diluted (6:10) in PBS 1X), in a 50 ml polycarbonate tube. The tubes were centrifuged at 400 g at 4°C for 5 min. The supernatant was discarded and the pellet was washed once with PBS. The cells were suspended in growth medium to determine viability.

Cell culture

Cells were plated on polystyrene dishes at a density of $4.5\text{--}1\times 10^5$ viable cells/cm², in growth medium with 10% of FBS, and cultured at 37°C with 5% CO₂. After 2 or 3 days, flasks were checked for contamination and medium was changed. Thereafter the cells were observed and medium was changed twice a week. On the third change of medium, the serum was reduced to 5%.

Immunocytochemistry

Culture dishes were washed with Dulbecco's PBS and fixed with formaldehyde 10% in PBS at room temperature. On the day of use, the cells were washed in water for 5 min and then in PBS for 15 min at room temperature to remove formaldehyde.

Inactivation of endogenous peroxidase activity was performed by incubating cells with H₂O₂ (30%) 10% in PBS at room temperature for 30 min. Non-specific binding was blocked with protein Block solution at room temperature for 20 min, before incubation with the primary antibody. The dishes were then incubated with anti- α actin (1:100), or anti-BSA (diluted 1:10) or PBS for control. These incubations were carried out in a wet camera at room temperature for 1 h, then at 4°C overnight and again at room temperature for 1 h. The cells were then incubated with a biotinilated second antibody Multilink at room temperature, for 1 h, and with peroxidise conjugated Avidin (1:20) at room temperature, for 1 h. After every step, the cells were washed with PBS, for 15 min. The immunoreactive sites were visualised with the addition of DAB chromogenic-substrate system prepared just before its use. After 5 min the reaction was stopped with distilled water, and cell nuclei were stained with Hematoxiline.

Metabolic studies

7-EC was used to assess the drug-metabolising capacity of the cultures. For each experiment, incubations with 25 μ M of 7-EC were performed. Biotransformation from 7-EC to 7-OH were quantitated using external

standard curves of 7-OH. A previous enzymatic reaction was used to transform the 7-OH glucuronide and 7-OH sulfate into 7-OH. Briefly: 1 ml of β -glucuronidase 2 mg ml⁻¹ in 1.25 M acetate buffer, pH 5, with 1 ml of medium were incubated overnight at 37°C. Metabolites were then extracted three times with 500 μ l of chloroform and then evaporated under N₂, and finally re-dissolved with 100 μ l of 0.5 M glycine buffer, pH 11. HPLC analysis was performed using a Hewlett Packard 1050 system, quaternary pump, with Hypersil column 3 μ m ODS 4.6 $\times$ 150 mm, from Phase Separations, and guard column Wipore C₁₈ ODS 4 $\times$ 3 mm, from Phenomenex. The mobile phase comprised of methanol, 10% acetic acid and water, in the following gradient: 20:2:88, 0–2 min; 60:2:38, 10–15 min; at a flow rate of 1 ml/min, 35°C. The detection was made by UV absorption at 320 nm. Under these conditions retention times for 7-OH and 7-EC were 8.2 and 13.0 min respectively.

Results

Culture of bovine hepatocytes

The yield of viable cells was $(1.60\pm0.57)\times10^8$ cells per gram of tissue. When no purification was made the cell population was not homogeneous, and fibroblast-like cells covered most of the surface of the dish. Because of their faster growing rate, confluence had been achieved in less than 2 weeks. The experience with calves and steers as donor animals, leads us to conclude that cellular proliferation is higher in calves.

When the Percoll purification step was performed, only larger cells were selected with a lower yield: $(1.76\pm0.95)\times10^7$ cells per gram of tissue. In this case, after 2 weeks of culture only some isolated colonies were seen on the dish. In the following weeks, even though the monolayer was not confluent, the clusters were large enough to perform immunocytochemical reactions.

Microscopic appearance of hepatocytes. Phase contrast microscopic observation of cells

Hepatocytes on day 18 are shown in Fig. 1a. The cells exhibit well-defined membranes, nuclei, nucleoli and a finely granular cytoplasm.

Without the purification, smaller cells were displayed, with more elongated morphology and less distinct nuclei (Fig. 1b).

Anti-BSA. Anti- α -actin

Cells expressing their albumin synthesis capability could only be seen when the purification step was included in the procedure of isolation (Fig. 2a).

Most cells in non-purified cultures exhibit positive reaction to α -actin, but in pre-purified cultures positive cells were not found (Figs. 2b, 3a).

Metabolism of 7-ethoxycoumarin

In order to determine the functionality of P450 activity we studied 7-EC metabolism in the cell cultures. Data obtained after 96 h incubation, in 1 day-old cultures or cultures at 80% confluency cultures, are presented in Table 1. While non-purified cultures exhibited a

Fig. 1 Phase contrast photomicrographs of bovine cells. Cells isolated after Percoll purification 15 $\times$ (a), and cells isolated without Percoll purification 50 $\times$ (b)

Fig. 2 Immunocytochemical reactions. Cells isolated after Percoll purification. Anti-BSA 25 $\times$ (a), Anti-Actin 10 $\times$ (b), Control 25 $\times$ (c)

higher yield of viable cells and reached confluence earlier, they presented lower metabolic activity, when evaluated as 7-EC metabolites formed per cell per hour. Close to confluence, purified cultures still presented a higher activity, though the amount of metabolites formed was lower.

Discussion

The hepatocyte isolation method shown in this work is inexpensive and easy to apply.

Fig. 3 Immunocytochemical reactions. Cells isolated without Percoll purification. Anti-Actin 25 $\times$ (a), Control 25 $\times$ (b)

We characterized cell type using anti- α -actin reaction on the cultured cells. Actins are protein constituents of the microfilaments, which are parts of the cytoskeleton and are a useful marker for Ito cells (Geerts et al. 1998).

After the separation of parenchymal cells, cultures of bovine hepatocytes retained their phenotypical characteristics. Production of albumin is a useful marker of synthetic liver-specific function. Most serum albumin is synthesized in the liver (Berry et al. 1991; Funaki et al. 2002). Immunochemically, albumin can be localized within hepatocytes, presumably at the site of synthesis.

The primary cultures of hepatocytes should be seeded in low density on collagen or fibronectin covered dishes to favour growth related activities (Nakamura et al. 1983a, b). The proliferation and differentiation of cells in a culture are highly affected by their culture environment, including the attachment substrate (Enat et al. 1984; Lorenti et al. 2001). In order to increase the lifetime and functionality of cells some authors have employed co-cultures of hepatocytes with epithelial or fibroblasts cells (Bhatia et al.

Table 1 7-Ethoxycoumarin metabolism

	Metabolites formed (nmol/h/10 ⁶ cell)	
	Purified cell cultures	Non-purified cell cultures
1 day-old	128.31±4.29	24.97±1.98
80% confluency	32.33±3.01 ^a	0.31±0.06 ^b

^a 31 day-old cell cultures^b 23 day-old cell cultures

1998). Here we report the culture of normal bovine hepatocytes on polystyrene flasks, without any exogenous matrix, even after separation of hepatocytes from other different liver cell types. Under these conditions the cultured cells reach confluence after 3–4 weeks, depending on the age of the animal, that is to say, shorter periods for younger animals.

Liver preparations are commonly used for *in vitro* metabolism studies of biotransformations, such as couple phases I and II. A substrate capable of undergoing oxidative and conjugative pathways of metabolism (7-ethoxycoumarin) was used as a marker of metabolic competence of a number of *in vitro* systems (Fry et al. 1976; Shull et al. 1986). Therefore, these cultures were capable of dealkylating 7-ethoxycoumarin. The low reaction yield near confluence was probably due to the lack of any stimulatory factor like NADPH (Berry et al. 1991). The non-purified cultures exhibited lower metabolic activity than purified ones (expressed as nmol of 7-OH formed per hour per cell). That could be explained by taking into account that most cells were non-parenchymal ones. The results with 7-EC metabolism suggest that purified cultures may be more suitable for their use in xenobiotic metabolism studies.

This simplified procedure for the isolation of functional and viable hepatocytes can be used as a starting point for setting up hepatocytes for *in vitro* comparative studies.

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