Cytotechnology (2008) 58:85–92
DOI 10.1007/s10616-008-9169-5

ORIGINAL RESEARCH

Optimization of the isolation and cultivation of *Cyprinus carpio* primary hepatocytes

Fan Yanhong · He Chenghua · Liu Guofang · Zhang Haibin

Received: 13 May 2008 / Accepted: 18 September 2008 / Published online: 18 October 2008

Abstract The aquatic environment is affected by numerous chemical contaminants. There is an increasing need to identify these chemicals and to evaluate their potential toxicity towards aquatic life. In this research we optimized techniques for primary cell culture of *Cyprinus carpio* hepatocytes as one adjunct model for ecotoxicological evaluation of the potential hazards of xenobiotics in the aquatic environment. In this study, *Cyprinus carpio* hepatocytes were isolated by mechanical separation, two-step collagenase perfusion, and pancreatin digestion. The hepatocytes or parenchymal cells could be separated from cell debris and from non-parenchymal cells by low-speed centrifugation (Percoll gradient centrifugation). The harvested hepatocytes were suspended in DMEM, M199 (cultured in 5% CO_2), or L-15 (cultured without 5% CO_2) medium then cultured at 17, 27, or 37 °C. Cell yield was counted by use of a hemocytometer, and the viability of the cells was assessed by use of the Trypan blue exclusion test. Results from these studies showed that the best method of isolation was pancreatin digestion (the cell yield was 2.7×10^8 per g (liver weight) and the viability was 98.4%) and the best medium was M199 (cultured in 5% CO_2) or L-15 (cultured without 5% CO_2). The optimum culture temperature was 27 °C. The primary hepatocytes culture of *Cyprimus carpio* grew well and satisfied requirements for most toxicological experiments in this condition.

Keywords Hepatocytes · Primary culture · Cell viability · *Cyprimus carpio*

F. Yanhong · H. Chenghua · L. Guofang · Z. Haibin (✉)
College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
e-mail: haibinzh@njau.edu.cn

Introduction

The increased use of synthesized chemical compounds in recent decades has brought with it the need to develop rapid, sensitive, reproducible, and cost-effective methods to assess toxicity before production. Environmental contaminants in the aquatic environment have necessitated the development of tools for environmental biomonitoring. Cells are a key level of organization for understanding mechanisms of toxicity. In coping with the new approach to risk assessment for regulatory tests and satisfying the social desire to reduce or replace the use of animals in testing (Castano et al. 2003), cultured cells have been applied as alternative models in mechanistic studies and toxicity identification in ecotoxicology. Fish are the dominant vertebrate species for regulatory evaluation of ecotoxicology and are also accorded the same legal protection as model mammals.

Fish cells have many functions that are similar to those of mammalian cells, but they also have many advantages over mammalian cells. For example, they

can be cultured at room temperature (20–28 °C) and can be directly exposed to environmental samples of different osmolarity. This means they greatly facilitate the collection of more useful in-vitro data for toxicity tests. Indeed, over 150 cell lines have been established from fish. The advantages of these cell lines are that they are standardizable, easy to handle with relatively low variability, more convenient, and less laborious. However, the limitations are that they are less differentiated and have lost most of their original genetical/biochemical characteristics. In contrast, primary cultured cells keep most of their original characteristics and can be used as a bridge between cell lines and in-vivo systems. Moreover, primary cultured cells are considered to be more sensitive, with high metabolic capacity compared with cell lines (Chen et al. 2003).

The technique used routinely for high-yield isolation of vertebrate liver cells is two-step collagenase perfusion, originally described by Berry and Friend (1969). Since then, use of isolated hepatocytes has provided an ideal system for evaluating many aspects of hepatic metabolism, including biochemical and cellular processes involved in the activation of toxic chemicals and environmental pollutants (Berry et al. 1991; Baksi and Frazier 1990; Guillouzo 1998; Kelly et al. 1998; Moon et al. 1965; Rogiers et al. 1995).

Mammal primary culture techniques (for example mechanical separation and pancreatin digestion) have been adapted to piscine species with various degrees of success by researchers who have used freshly isolated or primary cultured hepatocytes (Birnbaum et al. 1976; Braunbeck and Segner 2000; Campbell et al. 1983; Ha yashi and Ooshiro 1985; Ferraris et al. 2002; Klaunig 1984; Moon et al. 1965; Mommsen et al. 1994; Pesonen and Andersson 1991; Segner 1998; Riol et al. 2001). The classical method of primary culture of fish hepatocytes is whole-liver perfusion. As far as we are aware, Birnbaum et al. (1976) were the first to adapt the perfusion technique for preparing fresh suspension of fish liver cells and methods for primary culture of fish hepatocytes became available during the 1980s (Blair et al. 1990; Klaunig et al. 1985). This tool has been increasingly applied to physiological and toxicological studies, and has become the classical method (Braunbeck and Segner 2000; Mommsen et al. 1994; Monod et al. 1998; Pesonen and Andersson 1991; Segner 1998). Although the average cell yield by this method is 1–3 $\times$ 10^8 per g of liver weight, with a viability (Trypan blue assay) of 85% (Ferraris et al. 2002), it requires a perfusion machine and wastes a large amount of collagenase. Some researchers have tried to use pancreatin digestion to separate the hepatocytes (Liang yue et al. 2006). The method does not require special equipment or a large amount of pancreatin, and the cell yield can be increased to 10^8 per g of liver weight, but the viability of harvested hepatocytes is lower (Liang yue et al. 2006). Another method is mechanical separation. In this approach, a small amount of tissue is enough, but cell yield is low (10^5 per g of liver weight) and the separation process can cause more injury to the cell (viability 78.3%) (Braunbeck and Segner 2000).

Dulbecco's modification of Eagle's medium (DMEM) is the most common medium for cell culture. Medium 199 (M199) is used as the classical medium for culture of fish hepatocytes (Schreer et al. 2005). L-15 medium is a special medium that is used without 5% CO_2 to balance the pH, so it can be used in a conventional incubator. In this study, we chose these three media for our research to try to find the optimum medium for culture of *Cyprinus carpio* hepatocytes.

In mammal cell primary culture the optimum temperature is 37 °C. In aquatic animals, most of its living environment is 15–30 °C. We therefore used three culture temperatures (17, 27 and 37 °C) to find the optimum culture temperature.

Up to now, there is no study to show which is the best separation method, culture medium, and culture temperature during the aquatic cell culture process. This paper presents results from our attempts to optimize these procedures for isolation and cultivation of *Cyprinus carpio* primary hepatocytes.

Materials and methods

Fish

Cyprimus carpio weighing 100–150 g were obtained from a local hatchery, and were kept in a 1,000-L tank containing municipal potable water, They were acclimatized for two weeks before being used for experiments. During this time they were fed with commercial food, three times every week. Water temperature was maintained at 25 $\pm$ 1 °C, and dissolved oxygen was maintained between 7.1 and

7.6 mg L^{-1}. Fish were exposed to a 12-h light/12-h dark photoperiod, and the tank water was changed once every five days.

Cell isolation

Mechanical separation

After opening of the abdomen the liver surface was washed with sterile DBSS (composition given in Table 1). The liver was then cut into 1.0–2.0 mm^3 pieces by use of sterile stainless-steel eye scissors and forceps. The pieces were placed in a cool dish containing DBSS. The liver mass was washed three times with cooled DBSS then clipped into fragments. The fragments were placed on a stainless-steel mesh (inner diameter 0.3 mm) and gently crushed through the mesh. The tissue suspension was collected, placed on another stainless-steel mesh (inner diameter 0.075 mm), and crushed through the mesh. The liver cells were collected by low-speed centrifugation (120 × *g*; Percoll gradient centrifugation) and centrifuged three times in medium (DMEM, M199, or L-15; with 10% fetal bovine serum). The cell pellets were washed twice in the same medium supplemented with 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cell yield was counted by use of a hemocytometer and the viability of the cells was assessed by use of the Trypan blue exclusion test.

The media used in this work were obtained from Gibco. The reagents used in the work were general reagents purchased from Sigma.

Two-step collagenase perfusion

The abdomen was opened, the portal vein cannulated, and the first perfusion was performed in situ with Buffer I (composition given in Table 1) at a flow rate of 2 mL min^{-1}. After the blood had been cleared, pre-perfusion was performed at an initial flow rate of 5 mL min^{-1} with Buffer II (composition given in Table 1) (Hyllner et al. 1989; Klaunig et al. 1985). Then, following collagenase digestion of the liver, it was transferred to a chilled dish containing medium (DMEM, M199, or L-15; with 10% fetal bovine serum). The liver was then teased with stainless-steel forceps and shaken gently on ice for 30 min. The cells were then dispersed and filtered through a stainless-steel mesh (0.075 mm), collected by low-speed centrifugation (120 × *g*, Percoll gradient centrifugation), and centrifuged three times in ice-cold medium (DMEM, M199, or L-15). The cell pellets were washed twice in the same medium supplemented with 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cell yield was counted by use of a hemocytometer, and the viability of the cells was assessed by use of the Trypan blue exclusion test.

Pancreatin digestion

The abdomen was opened and the liver surface was washed with sterile DBSS. The liver was then cut into 1.0–2.0 mm^3 pieces by use of sterile stainless-steel eye scissors and forceps and the pieces were placed in a cool dish containing DBSS. The liver mass was

Table 1 Composition of the solutions used for preparing hepatocytes

Component	Buffer I	Buffer II	DBSS	0.1% pancreatin digestive
NaCl (mM)	126.00	126.00	126.00	126.00
KCl (mM)	4.82	4.82	4.82	4.82
$CaCl_2$ (mM)	–	1.50	–	–
$MgSO_4$ (mM)	0.60	0.60	–	–
$NaHCO_3$ (mM)	3.60	3.60	–	–
NaH_2PO_4 (mM)	3.30	3.30	–	–
Na_2HPO_4 (mM)	8.90	8.90	6.1	3.2
KH_2PO_4 (mM)	–	–	1.5	1.5
EDTA (mM)	10.00	–	–	0.54
HEPES (mM)	–	–	21.9	–
Pancreatin (g/L)	–	–	–	1
Collagenase (U mL^{-1})	–	0.04–0.14	–	
pH	7.2–7.4	7.2–7.6	7.2–7.6	

washed three times with cooled DBSS, then clipped into fragments. Pancreatin digestive juice (0.1%; composition given in Table 1) was added to the dish to digest the fragments for 30 min. During the digestion the fragments were blown gently by use of a Pasteur pipette. The digested fragments were then filtered through a stainless-steel mesh (0.075 mm) into medium (DMEM, M199, or L-15; with 10% fetal bovine serum). The cells were collected by low-speed centrifugation (120 × *g*, Percoll gradient centrifugation) then centrifuged three times in medium (DMEM, M199, or L-15; with 10% fetal bovine serum). The cell pellets were washed twice with the same medium supplemented with 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The cell yield was counted by use of a hemocytometer and the viability of the cells was assessed by use of the Trypan blue exclusion test.

Plating and cell culture

Cells were plated at a density of 2×10^6 mL^{-1} viable cells, 200 μL/well in 96-well plates. Before use, the 96-well plates were coated with 0.1% gelatin for 30 min and washed twice with serum-free medium. The 96-well plates were incubated at 17, 27, or 37 °C for 1 week. The medium was changed after incubation for 72 h.

The modes of cell culture used in this study are shown in Fig. 1. Cells cultured with DMEM and M199 media were kept in a CO_2 incubator which provided 5% CO_2; those cultured in L-15 medium were kept in a conventional incubator without 5% CO_2.

Microscopy

After 72 h incubation, inverted light microscopy was used to observe the condition of the cells. At the same time, the medium was changed. Subsequently the condition of the cells was observed every 24 h.

MTT cell viability assay

The medium was moved carefully from the wells and the wells were washed once with PBS. MTT (concentration 5 mg mL^{-1} in PBS; 20 μL/well) was then added to the wells. The cultured cells were returned to the incubator for 4 h. The medium was then discarded and the wells were dried. Formazan crystals produced were solubilized using 100 μL dimethyl sulfoxide (DMSO)/well and misce bene. Absorbance was measured in a Stat Fax 2000 ELIASA (Bio-Rad, USA) at 570 nm wavelength, to assess cell proliferation of the primary hepatocytes. Cell growth was monitored over a one-week period using the MTT assay (Mosmann 1983).

Results

Cell yield and viability

When we chose the mechanical separation method, the cell yield was 1.3×10^5 per g (liver weight) and the viability was 87.3%. In the two-step collagenase perfusion method the cell yield was 6.1×10^6 per g (liver weight) and the viability was 96.7%. When using the Pancreatin digestion method, however, the

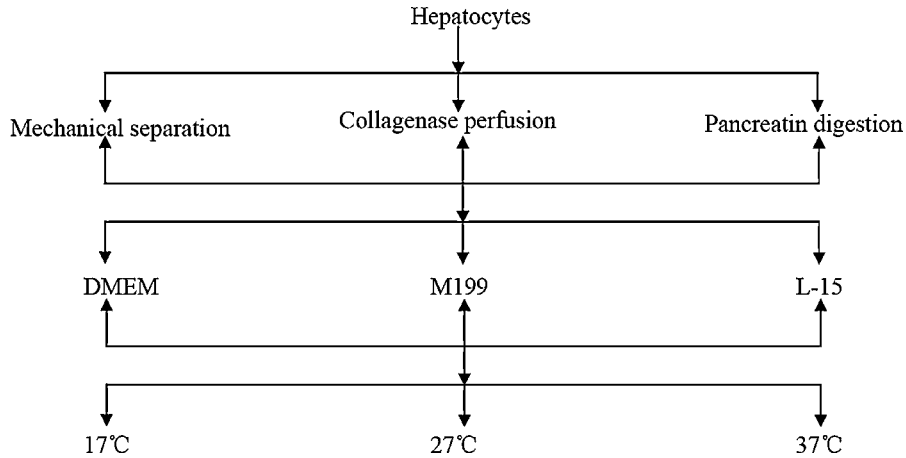

Fig. 1 Modes of hepatocyte culture and isolation in this research

cell yield was 2.7×10^8 per g (liver weight) and the viability >98.4%. Irrespective of the method selected to isolate the hepatocytes, hepatocyte purity was >95.1% as determined by microscopic morphology after Percoll gradient centrifugation.

Microscopy

A single cell suspension was distributed uniformly over the tissue culture plates. Within 30 min the cells began to adhere to the substrate and after 24 h cells were firmly attached in aggregates to the Matrigel-coated plates (Fig. 2).

The hepatocytes cultivated at 27 and 17 °C grew well and were monitored for cellular changes after 72 h incubation. Most of the cells cultivated at 37 °C floated in the medium after 72 h incubation and few cells survived. The quantity of anchorage-dependent cells in DMEM was less than that in M199 and L-15 but the difference for cells cultured in different environments was not significant ($p > 0.05$).

MTT reduction assay

The primary hepatocytes cultivated at 27 °C grew better than at 17 and 37 °C as demonstrated by the MTT assay (Fig. 3). If we chose separation method as the single factor, the primary culture hepatocytes grew better by the pancreatin digestion method than by mechanical separation or two-step collagenase perfusion. If we selected the medium as the only factor, we could see that the tendency of cell growth was similar and that the hepatocytes grew better in L-15 than in DMEM and M199. In conclusion, we believe the optimum process for isolation and cultivation of *Cyprinus carpio* primary hepatocytes was pancreatin digestion, culture in M199 (cultured in 5% CO_2) or L-15 (cultured without 5%CO_2), and culture temperature 27 °C.

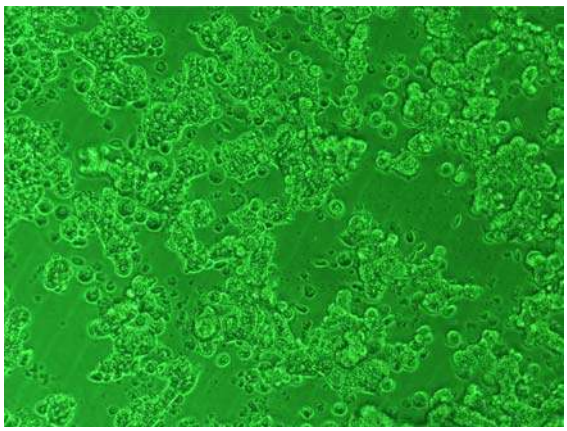

Fig. 2 Primary culture hepatocytes attached to the Matrigel-coated substrate after 24 h in culture (200×)

Discussion

During the 80s, application of freshly isolated fish hepatocytes was greatly expanded. Rather few studies explored the use of primary cultures instead of fresh isolates, a fact which might be related to technical problems with in-vitro maintenance of fish hepatocytes. Requirements for a primary culture system for hepatocytes include an optimized isolation procedure and the provision of optimum culture conditions (separation method, medium, and temperature) for survival and functioning of the cells.

Mechanical separation was used in the early period. It is widely used in cell microculture because of the small amount of tissue required. We do not recommend this procedure unless it is necessary, because it can cause injury to the cell and reduce the cell yield and viability during the disassociation process. Two-step collagenase perfusion is the classical method of obtaining not only mammalian hepatocytes but also those of teleostean fish (Segner 1998). In this study we tried the pancreatin digestion method to isolate hepatocytes. We reduced the concentration of pancreatin and added EDTA to the digestive juice. EDTA can move Ca^{2+} and Mg^{2+} from the cell junction and so can facilitate the cell-isolation process. And at the same time, the lower concentration of pancreatin could lessen injury of the hepatocytes. Maybe this was the reason the viability of the hepatocytes was high by this method in our research.

Cell suspensions obtained after perfusion of teleost fish liver contained a variety of cell types (for example biliary epithelial cells and macrophages; Braunbeck and Segner 2000). Approximately 10% of the cells isolated from carp liver were pancreatic cells. These could be removed by density gradient centrifugation (Bounche et al. 1979) or they might be cultured together with the hepatocytes (Mitteregger et al. 1999). The presence of pancreatic cells has no

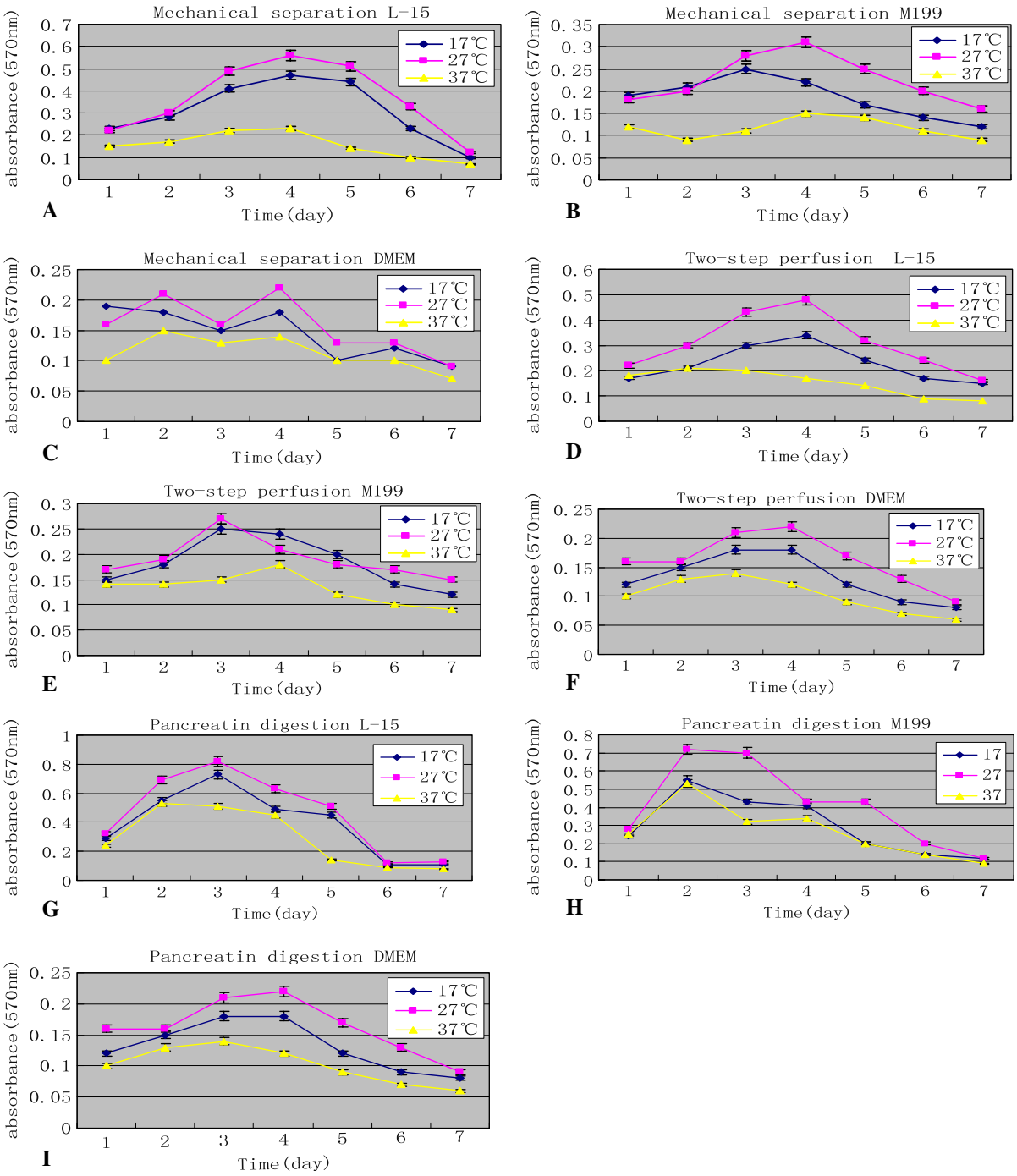

Fig. 3 Results from assessment of the viability of primary culture hepatocytes by use of the MTT assay. (**a**), (**b**), (**c**), mechanical separation and cultured in L-15, M199, or DMEM, respectively; (**d**), (**e**), (**f**), two-step perfusion and cultured in L-15, M199, or DMEM, respectively; (**g**), (**h**), (**i**), pancreatin digestion and cultured in L-15, M199, or DMEM, respectively. Each point is the mean from six experiments

negative effect on cultured hepatocytes, probably because the pancreatic cells release negligible amounts of proteolytic enzymes into the medium. In our study we used density gradient centrifugation to obtain high-purity hepatocytes and tried to find a better way to isolate the hepatocytes from the liver.

Cell yields from liver vary with species, strain, sex, and the age of the donor fish (Braunbeck and Segner 2000); Yields ranged from 30% to 90% of initial liver weight (Braunbeck and Segner 2000). After collagenase perfusion the average yield of hepatocytes from most fish species was about 10^8 viable cells per g liver. In our research, cell yield was 6.1×10^6 per g (liver weight) and 2.7×10^8 per g (liver weight) when we chose the two-step collagenase perfusion method and pancreatin digestion method, respectively.

The cell number seeding each well was the same in our research, so their growth tendency was similar after seeding for 24 h. The viability of the cells was different, however. One difference from mammals is that the optimum growth temperature of primary culture *Cyprinus carpio* hepatocytes was 27 °C not 37 °C. The growth temperature of *Cyprinus carpio* is 15–25 °C, and this may be the reason for the difference. The hepatocytes grew well at 17 °C and were also satisfactory for toxicological testing. At 37 °C, cells viability was low, and unfit for the next experiment. (It was also observed that primary culture fish hepatocytes died quickly at 37 °C).

In this study we chose three media (DMEM, M199, and L-15). DMEM is the basal medium of cell culture and is widely used in mammalian cell-culture processes. M199 medium is an optimum medium for culture of *Cyprimus carpio* hepatocytes (Schreer et al. 2005). In our early research, however, we found it seemed likely that fish hepatocytes could be cultured without 5% CO_2. For this reason we also selected the L-15 medium. In our study *Cyprinus carpio* hepatocytes cultured in M199 or L-15 media were sufficiently viable for toxicology study. We found in this research that cells had the best viability in L-15 medium and that culture of *Cyprinus carpio* hepatocytes with 5% CO_2 was not essential. Because cultivation in a CO_2 incubator is not necessary, the method could expand application of cell culture technique. We recommend L-15 medium as an optimum medium for culture of *Cyprinus carpio* hepatocytes.

Fish primary hepatocyte cultures have been used to detect P4501 A1-inducing compounds in organic fractions extracted from pulp and paper mill effluent (Pesonen and Andersson 1992) and in fractions extracted from Baltic salmon (Hakansson et al. 1991). Gagne et al.(1995) used trout hepatocytes to detect persistent genotoxin compounds in a sediment extract obtained in the vicinity of a creosote-treated wharf, and compared results with chemical analysis for PAHs in the same sediment. Fish hepatocytes could therefore be used in a screening system to identify chemicals that interfere with hormone response and to study cellular mechanisms behind reproduction impairment. This is a particularly important area of research, because several species of fish-eating birds and fish have been shown to exhibit chronic impairment of reproduction.

In this research we have optimized the isolation procedure and culture conditions (pancreatin digestion, L-15 medium, and cultured at 27 °C). Our findings suggest that this primary cell system would be a useful aquatoxicological tool to screen for chemical contaminants.

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