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Preparation of Functionally Active Immobilized and Perfused Mammalian Cells: an Example of the Hepatocyte Bioreactor

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Summary

In the present study, a method has been employed for hepatocyte immobilization in agarose threads which allows for cell perfusion. The rat hepatocytes are isolated from the liver. A 1.8 % low-gelling agarose solution is prepared in warm Krebs-Henseleit solution. The agarose solution is mixed 1:1 with the hepatocytes and the cells are immobilized in agarose threads by extruding the agarose-cell mixture through cooled Chemfluor teflon (TFE) tubing. Light and electron microscopy studies indicated the integrity of the hepatocytes in the gel matrix. This system allows for liver cell perfusion and viability studies to be carried out non-invasively on the cells and provides data that are comparable to those obtained with a perfused isolated liver. Immobilized hepatocytes are an *in vitro* system worthy of further evaluation which may be useful in the studies of liver cell metabolism and the response of the liver to foreign chemicals.

Key words

Hepatocytes – Immobilization – Perfusion

Introduction

Isolated hepatocytes are now being studied in an extensive number of investigations in biochemistry, physiology, molecular biology, pharmacology and other areas. This experimental cellular model serves as an intermediate system between studies of the whole animal or the isolated perfused liver, on one hand, and those with isolated organelles or solubilized enzymes, on the other (Moldeus *et al.* 1978). In a previous article on this isssue we pointed out the importance of the immobilization and perfusion of mammalian cells for a better function of cells, as compared with the so-called static cellular system. This article illustrates the application of this technique, by using isolated rat liver cells, for the preparation of a hepatocyte bioreactor. The cellular functionality and integrity in the gel matrix was monitored, both biochemically and histologically.

Before giving the description of how a hepatocyte bioreactor can be prepared, we would like to mention the first trials by Van der Meer and Tager (1976) and Cohen *et al.* (1978) in using isolated liver cells for perfusion experiments. The latter authors described simple methods for the perfusion of isolated liver cells without immobilization. One major difficulty of these techniques was the selection of an appropriate filter for the separation of cells and the perfusate. Such a filter should not clog in the course of the perfusion and the intact cells should not pass through the filter. Later on, a number of reports on various methods of cell immobilization for the perfusion studies, using either animal or plant cells, for NMR spectroscopy as well as other types of metabolic studies have been published (Gillies *et al.* 1986, Brodelins *et al.* 1987, Foxall and Cohen 1983).

The most widely applied and well developed technique, so far, is the immobilization of cells in threads of agarose. In the present investigation, a method developed Foxall and Cohen (1983, 1984) has been employed for hepatocytes immobilization in agarose threads which allows for cell perfusion up to and beyond 24 hours. This hepatocyte system is readily

perfusable under a variety of conditions and has been shown to be useful as a convenient model for assessing various liver functions. Among others, the cells were tested for the hepatotoxicity of a wide variety of putative hepatotoxic agents (Farghali *et al.* 1991, 1992).

The technique involves mixing of a dense hepatocyte suspension with an equal volume of agarose solution. The resulting slurry is passed through an ice bath, where the agarose solidifies, and is extruded into a sample tube through a cannula. The resulting agarose thread is then perfused with fresh media. Using this system, cells can remain viable for relatively long time and a variety of important parameters can be monitored.

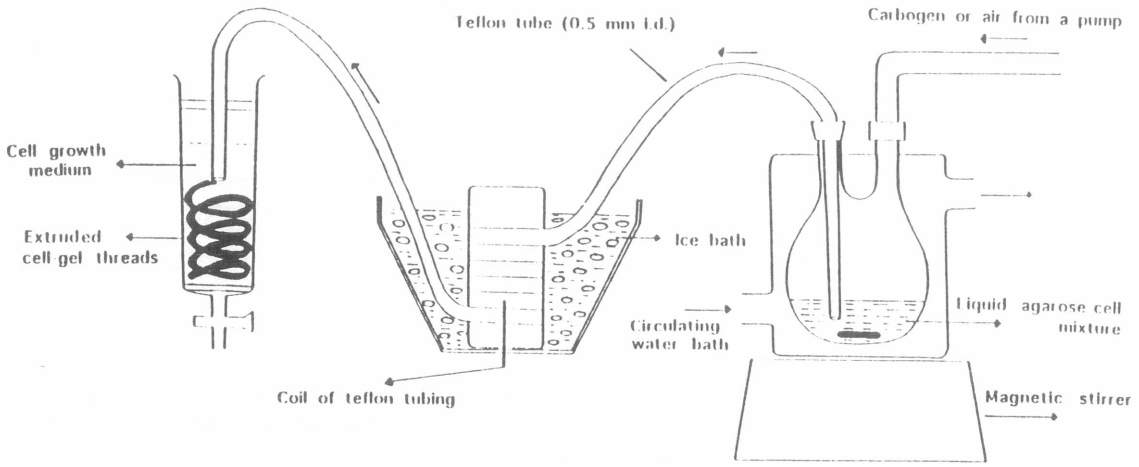

Fig. 1
Block diagram of the apparatus required to embed hepatocytes within threads of agarose gel (modified from Foxall *et al.* 1984)

Methods and Results

Procedure for the immobilization of hepatocytes in agarose threads

The rat hepatocytes are isolated from the liver by a standard two-phase perfusion method. The first phase includes perfusion of the liver with a calcium- and collagenase-free medium, while in the second phase calcium and collagenase are being added, as reported earlier (Moldeus *et al.* 1978). A 1.8 % low-gelling agarose solution is prepared in a warm Krebs-Henseleit (KH) solution at 70 °C. The agarose solution is brought to 37 °C. The thermostated agarose solution is mixed 1:1 with the hepatocytes at a density of 3–6 x 10^7 cells/ml (viability not less than 90 %). The cells are immobilized in agarose threads by extruding the agarose-cell mixture through cooled Chemfluor teflon TFE tubing (0.5 mm internal diameter) into an appropriate tube containing RPMI 1640 medium under carbogen (95 % O_2 and 5 % CO_2). The threads are compressed gently to form a densely packed column as depicted in Fig. 1.

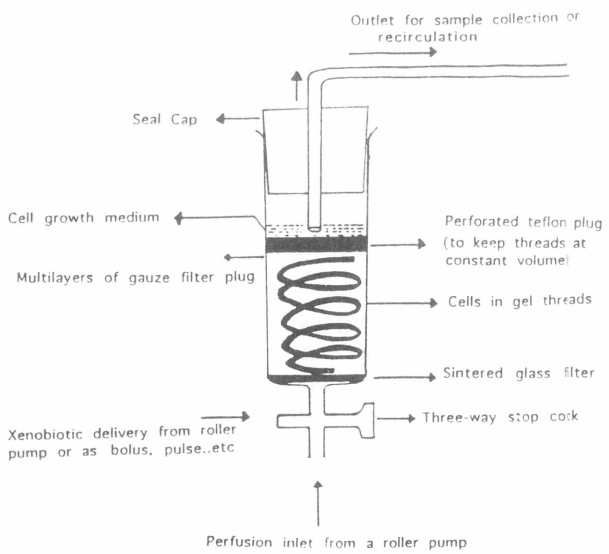

Fig. 2
Schematic representation of the tube used for perfusion of the cell-gel threads.

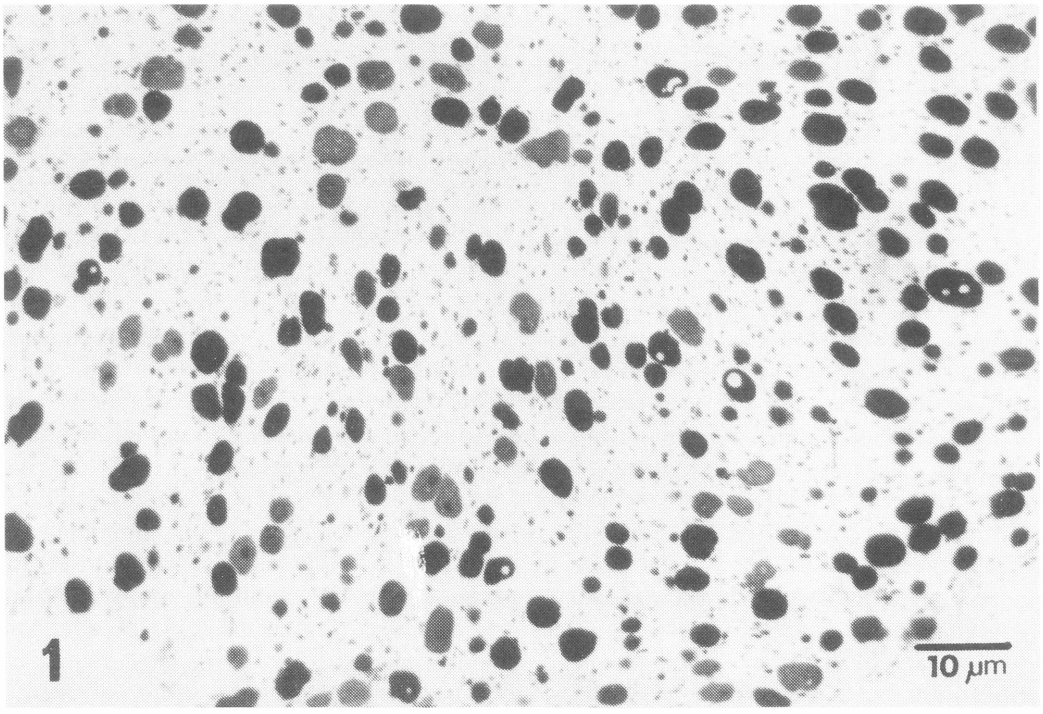

Fig. 3
A semithin section from the agarose thread demonstrates randomly regular distribution of hepatocytes in the bioreactor. Darkly stained cytoplasm of hepatocytes confirms the homogeneous ultrastructure of cells. Lightly stained cytoplasm (sometimes already vacuolized), corresponds to altered ultrastructure. Epon section, toluidine blue staining.

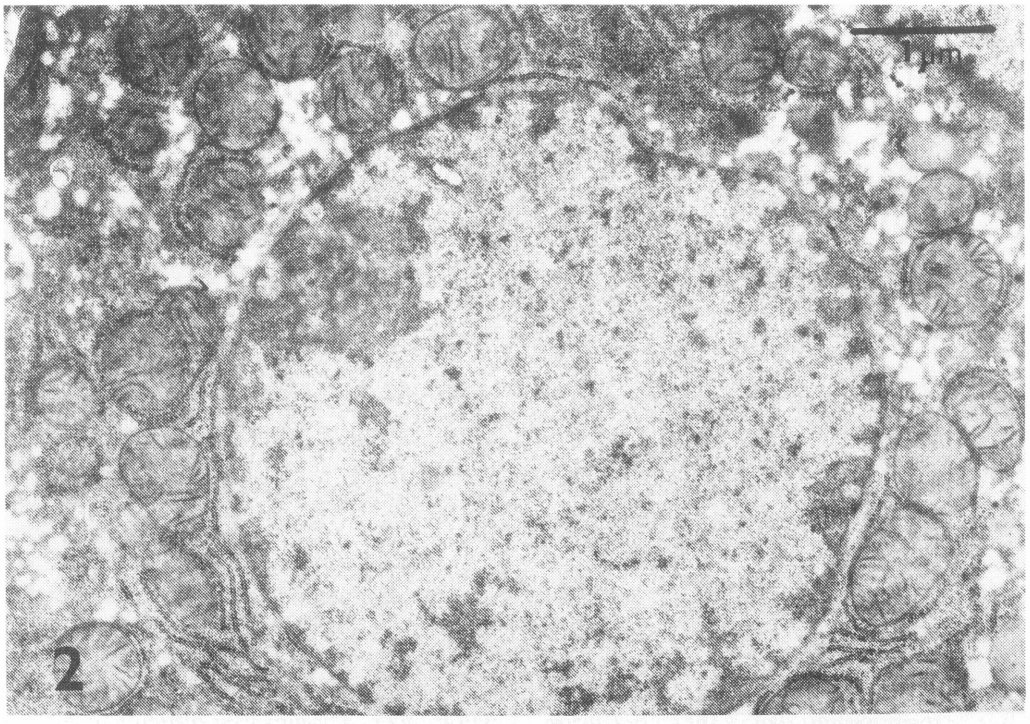

Fig. 4
A part of the hepatocytes from agarose thread. Note the finely granular chromatin structure of the nucleus, well preserved mitochondrial shape and structure as well as narrow formations of the granular endoplasmic reticulum. Slightly dilated vesicles of the smooth endoplasmic reticulum are the only difference from the classical ultrastructure of hepatocytes in ultrathin liver sections.

Perfusion of the threads and biochemical analyses

The threads are perfused with a thermoregulated and well-oxygenated (95 % O_2 and 5 % CO_2) RPMI 1640 medium inflowing from the bottom of the tube and outflowing through a tube fixed in a seal rubber cap at a rate of 10 ml/min in a non-recirculating system using a roller pump. The tube has two filters, one below and the second one above the hepatocytes agarose threads, to keep the latter in position. The upper filter device could be made of a nylon sieve with 110 μm pore size or several layers of medical gauze. This arrangement allows for the loose cells and debris to be washed out until the perfusate is clear, meanwhile restraining the cell-gel threads which are tightly kept in position by a perforated teflon plug (Fig. 2). The perfusion rate was selected on the basis of its ability to maintain a high ATP level within the hepatocytes (Farghali *et al.* 1992). In addition, it is known that under physiological conditions, hepatic blood flow ranges from 100–130 ml/min/100 g (Campra and Reynolds 1988). A second pump is used, if necessary, to deliver the compound of interest to the perfusate at a predetermined concentration using a three-way stopcock without affecting the final flow rate. Otherwise, the compound to be studied is either added as a bolus through the three-way stopcock located adjacent to the port of the inflowing perfusate to the immobilized cells or added directly to the perfusate reservoir. Before starting perfusion with the drug, the cellular system is equilibrated till a constant ^{31}P-NMR baseline spectrum and/or oxygen consumption rate and a minimum lactate dehydrogenase baseline are attained (about 60 min). These measurements are continued throughout the experiment.

Evaluation of cell function and integrity in the gel matrix

a) Cell viability in the gel matrix

Immobilized liver cell viability and function can be assessed periodically by:
(1) measuring O_2 consumption calculated from $(pO_2^{inflow} - pO_2^{outflow}) - pO_2^{blank}$ x flow rate (Fig 2);
(2) monitoring the percent of total leakage of lactate dehydrogenase from cells at regular intervals during the experiment;
(3) measuring the fructose phosphorylation achieved after a fructose bolus;
(4) cytologically by measuring, the trypan blue (0.5 %) exclusion after termination of the perfusion experiments. The cells are briefly removed from the gel thread by vigorous pipetting. The cell suspension is passed through a sterile nylon mesh (70 μm diameter) to remove the agarose debris. The cells are then diluted and tested for trypan blue exclusion (Knopp *et al.* 1984).

b) Histological evaluation of hepatocytes in the gel matrix

Isolated hepatocytes are almost regularly dispersed in the agarose threads in a density of 600–850 cells/mm^2. They are of nearly round or oval shape and their surface is furnished by irregular short microvilli at the electron microscopic level.

Semithin Epon sections (1 mm) stained with toluidine blue allow to identify a prevalence of cells with spherical nuclei containing predominantly euchromatin and large nuclei, indicating a high level of activity and viability (Fig. 3). This conclusion is also supported by electron microscopic findings of well developed formation of granular endoplasmic reticulum (GER), mitochondria and the Golgi complex (Fig. 4). On the contrary, the smooth endoplasmic reticulum is represented by predominantly dilated small vesicles. The remaining hepatocytes exhibit different signs of degeneration, indicated by a higher density of chromatin in their nuclei including extreme pycnotic forms. Findings of gradual alterations in mitochondrial inner structure, reduction of GER and vacuolization of cytoplasmic matrix confirm further different stages of this process.

Discussion

Although *in vivo* studies are probably the most important, valuable and easiest to interpret they are often either difficult or unethical to perform. As a result, an approach using various *in vitro* cellular systems that simplify the conditions by excluding uncontrollable factors present in situations *in vivo* has been used for assessing drug effects and metabolic pathways *in vitro* (Knopp *et al.* 1984).

One of the most promising cellular systems is discussed in this series of papers in which the cells are immobilized and perfused in a bioreactor model. Indeed, the optimal use of the bioreactor in biology is when the investigator is able to correlate information obtained *in vivo* with that obtained *in vitro* from intact functioning cells in a controlled environment. Within the time frame of our experiments, the hepatocytes in the used perfusion system remained viable as assessed by trypan blue exclusion and lactate dehydrogenase loss. Moreover, the value for oxygen consumption achieved with this system agrees quite well with reported estimates of oxygen consumption for the whole liver. Though it is difficult to estimate precisely the number of hepatocytes in a perfused whole liver from which NMR spectra are obtained in a given experiment, following the administration of a fructose load the increase in the phosphomonoester peak area ratio due to the formation of fructose-1-phosphate is roughly identical to that obtained when 5 x 10^8 cells are

immobilized in gel threads and subsequently perfused. The ATP content of isolated perfused hepatocytes was 45 nmol/10^6 cells, a value which is in good agreement with the value of 3.2–3.8 mmol/g of freeze-clamped whole rat liver.

According to the available literature, there are no data describing the homogeneity of hepatocytes and integrity of subcellular organelles in the hepatocyte-gel threads as presented in this study. Our study using light and electron microscopy indicated the integrity of hepatocytes in the gel matrix and that these cells have retained an almost normal architecture.

In conclusion, the hepatocyte bioreactor in which cells are immobilized in agarose threads is described. This system allows liver cell perfusion and viability studies to be performed non-invasively which does not harm the cells and can be performed in real time. Importantly, it provides data that are comparable to those obtained with a perfused isolated liver. Using this model, it can be shown that immobilized hepatocytes are an *in vitro* system worthy of further evaluation which may prove to be useful in the field of liver cell metabolism and responses of the liver to foreign chemicals.

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References

BRODELINS P., LILJA H., MOSBACH K.: Overview of immobilization techniques for cells/organelles. *Meth. Enzymol.* **135 B:** Academic Press, Orlando, FL 173–175, 1987.

CAMPRA J.L., REYNOLDS T.B.: The hepatic circulation. In: *The Liver Biology and Pathobiology,* M.I. ARIAS, W.B. JAKOHY, H. POPPER, D. SCHACTER, D.A. SHAFRITZ, (eds), Raven Press, New York, 1988, pp. 911–930.

COHEN S.M., OGAWA S., ROTTENBERG H., GLYNN P., YAMANE T., BROWN T.R., SHULMAN R.G.: ^{31}P nuclear magnetic resonance studies of isolated rat liver cells. *Nature* **273:** 554–556, 1978.

FARGHALI H., SAKR M., GASBARRINI A., WILLIAMS D.S., DOWD S.R., HO C., VAN THIEL D.H.: A biochemical and ^{31}-P NMR investigation of the effect of FK 506 and cyclosporine pretreatment on immobilized hepatocytes perifused with ethanol. *Transplant. Proc.* **23:** 2805–2808, 1991.

FARGHALI H., ROSSARO L., GAVALER J.S., VAN THIEL D.H., DOWD S.R., WILLIAMS D.S., HO C.: ^{31}P-NMR spectroscopy of perifused rat hepatocytes immobilized in agarose threads: application to chemical-induced hepatotoxicity. *Biochim. Biophys. Acta* **1139:** 105–114, 1992.

FOXALL D.L., COHEN J.S.: NMR studies of perfused cells. *J. Magn. Reson.* **52:** 346–349, 1983.

FOXALL D.L., COHEN J.S., MITCHELL J.B.: Continuous perfusion of mammalian cells embedded in agarose gel threads. *Exp. Cell Res.* **154:** 521–529, 1984.

GILLIES R.J., CHRESAND T.J., DRURY P.D., DALE B.E.: Design and application of bioreactors for analyses of mammalian cells by NMR. *Rev. Magn. Reson. Med.* **1:** 155–179, 1986.

KNOP R. H., CHEN C.W., MITCHELL J.B., RUSSO A., MCPHERSON S., COHEN J.S.: Metabolic studies of mammalian cells by ^{31}P NMR using a continuous perfusion technique. *Biochi. Biophys. Acta* **804:** 275–284, 1984.

MOLDEUS P., HOGBERG J, ORRENIUS S.: Isolation and use of liver cells. *Meth. Enzymol.* **52:** 60–71, 1978..

VAN DER MEER R., TAGER J.M.: A simple method for the perifusion of isolated liver cells. *FEBS Lett.* **67:** 36–40, 1976.

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