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Hepatocyte isolation from pig livers after warm ischaemic injury

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Abstract Hepatocyte cultures have been used extensively for a wide variety of physiological, pharmacological and experimental studies. The warm ischaemic period before isolation is kept to a minimum to achieve a high yield of cells isolated and a good viability for culture. We have recently introduced a new concept of liver resuscitation after warm ischaemia that is based on a 3-h reperfusion period with an improved perfusate and simultaneous dialysis. In this study, we applied the new technique for hepatocyte isolation from livers subjected to 80 min of complete ischaemia at 37 °C. Cell yield was improved by a resuscitating perfusion from 58% to 73% and viability from 39% to 76%.

Key words Hepatocyte isolation · Hepatocyte culture · Warm ischaemia · Liver perfusion · Dialysis · Liver support systems

Introduction

Hepatocyte cultures have been used extensively for a wide variety of physiological, pharmacological and experimental studies [1–3]. The collagenase liver perfusion technique is a standard method for isolation of viable hepatocytes, being introduced by Howard and Pesch [4] and modified by Berry and Friend [5] and Seglen [6, 7]. Experimental isolations of hepatocytes from whole liver perfusions are usually performed with rat livers. If larger quantities are to be obtained, hepatocyte isolation is carried out with pig livers [8]. In a few experimental isolations, even human livers have been used [9, 10]. The warm ischaemic period is kept to a minimum to achieve a high yield of cells isolated and a good viability for culture. Recently, we have developed a method of liver perfusion that allows the resuscitation of livers subjected to 80 min of warm ischaemia at 37 °C [11]. This method is based on a perfusion period of 3 h and improved perfusate. A major problem of liver perfusion after warm ischaemia in a recirculating system is the regulation of pH and electrolyte homeostasis. We, therefore, have integrated a simultaneous haemodialysis circuit [12].

The aim of this study was to develop a method for *hepatocyte isolation* from livers after 80 min of complete ischaemia at 37 °C. Comparison was made between hepatocyte isolation from livers after 80 min of warm ischaemia with a 3-h perfusion period (resuscitation group) and hepatocyte isolation directly after a warm ischaemic period of 80 min (without resuscitation).

Materials and methods

Organ harvesting

Landrace pigs of either sex were used as liver donors. The animals were killed by electrocution in a commercial abattoir and exsanguinated. One litre of whole fresh pig blood was collected and 5000 U heparin were added. Organ harvesting and liver preparation were carried out by standard techniques. A median thoracotomy and laparotomy was carried out 20 min after cardiac arrest. The heart, lungs, liver and bowel were removed en-bloc. The completely isolated liver was wrapped in a double plastic bag and immersed in a

37 °C water bath for a further 50 min of warm ischaemia. The portal vein, the hepatic artery and the common bile duct were cannulated 10 min before reperfusion. The cystic duct was ligated and the gallbladder drained. All livers were then weighed; the average weight was 470 g. The period of warm ischaemia at 37 °C was 80 min for all livers.

Liver perfusion and dialysis

The livers were immersed in perfusate. The portal vein and hepatic artery were connected to the perfusion circuit and perfusion was started. The perfusion circuit consisted of two Stöckert roller pumps and a Cobe CML disposable membrane oxygenator with a reservoir as used in heart-lung machines. The portal vein was perfused from the reservoir by gravity, and the hepatic artery was perfused by a pressure-controlled roller pump. The perfusion pressure in the hepatic artery was kept between 60 to 80 mm Hg, which produced a flow of 150 to 250 ml/min. The perfusion pressure in the portal vein varied between 5 and 15 cm water, being highest at the beginning of perfusion and settling down to a steady state after about 20 min of perfusion. The resulting flow into the portal vein was 2–2.5 ml perfusate per gram liver weight per minute. The Cobe membrane oxygenator was supplied with a mixture of 95% oxygen and 5% carbon dioxide. The simultaneous dialysis circuit consisted of two Stöckert roller pumps, a Gambro ALWALL GFS 12 fiber dialyser and a reservoir containing 10 l dialysate. The perfusate was pumped at a rate of 400 ml/min through the dialyser. On the other side of the membrane, dialysate was pumped at a rate of 1000 ml/min from the reservoir in a recirculating mode. The volume of perfusate and dialysate was kept constant by control and adjustment of pressures on both sides of the dialyser. Reperfusion and dialysis were started at the same time.

Perfusate

The volume of circulating perfusate was 4 l. It was prepared by adding 1 l of whole fresh pig blood, containing 5000 U heparin, to 3 l HPF3 solution, which is based on physiological electrolyte concentrations [13, 14]. Oncotic pressure was provided by a gelatine-derived polypeptide (haemaccel 35, Behringwerke, Marburg, Germany). Buffering was by a bicarbonate/CO_2 system; no adjustments in pH were made during perfusion. The osmolality was 310 mosm/kg. The system was primed with 5000 U heparin at the start of perfusion. An additional 5000 U heparin were added at 90 min of perfusion. We added 20 essential and non-essential amino acids, fructose and oleate to the basic composition of the perfusate as published recently [11].

Dialysate

The dialysate consisted of commercially available acid and basic bicarbonate concentrate and distilled water. The ratio of dilution was 1:1.78:42.22, respectively. Before each experiment, 10 l of fresh dialysate were prepared. The prime buffer of the dialysate was sodium-bicarbonate. The dialysate was adjusted to a pH of 7.4, Na^+, 140 mmol/l, K^+, 3 mmol/l, HCO_3^-, 37 mmol/l and glucose, 64 mg/dl. The osmolality was 296 mosm/kg.

Isolation

Hepatocytes were isolated by collagenase perfusion according to the "two-step" perfusion technique described by Seglen, which was modified to a "five-step" perfusion technique by Gerlach [15]. We used collagenase type IV (Sigma, St. Louis, USA), which is prepared for hepatocyte isolation from the crude collagenase of *Clostridium histolyticum*. Collagenase was used in a concentration of 0.8%. Isolation was carried out in the same perfusion apparatus as the normothermic perfusion, but without dialysis. The solutions used for perfusion were maintained at 37 °C and equilibrated with 95% oxygen and 5% carbon dioxide. An initial perfusion was carried out for 15 min with 3 l HPF3 solution without Ca^{++}/Mg^{++} and with 2 m*M* EDTA to remove erythrocytes and Ca^{++}. In the second step, livers were perfused for another 15 min with 3 l HPF3 solution without Ca^{++}/Mg^{++}, followed by a 2-min perfusion with 1 l HPF3 solution with Ca^{++}/Mg^{++}. Collagenase perfusion was performed with 1 l of Dulbecco PBS with 5 m*M* Ca^{++}/Mg^{++}, 2.2 g/l sodium bicarbonate, 15 m*M* glucose, penicillin/streptomycin and collagenase. A final perfusion step was carried out with cooled (4 °C) HEPES-buffered Williams-E solution with penicillin/streptomycin and amphotericin.

Incubation

The Glisson capsule was disrupted by the sterile finger fracture technique. The cell suspension was filtered by a 300-μm followed by a 100-μm nylon mesh and subsequently centrifuged in standard medium at 15 g twice to remove cell debris, damaged cells and non-parenchymal cells. Further purification of hepatocytes was achieved by a 1.065 g/ml Percoll gradient centrifugation [16]. After Percoll treatment, cells were centifuged in Williams-E standard culture medium at 15 g. Then, cell suspension was incubated at 37 °C in Williams-E solution with 10% fetal calf serum, L-glutamine, penicillin/streptomycin and amphotericin. PH was maintained at 7.4.

The quality of liver perfusion was assessed by analysis of transaminases, electrolytes, pH, urea and bile production. The viability of hepatocytes was judged first by cell yield in percentage of liver wet weight, second, by trypan blue exclusion test (%) and, third by in vitro cell adhesion testing. The trypan blue exclusion test was carried out prior to Percoll gradient centrifugation. Adhesion culture was maintained over 48 h.

Analysis

In the group with a resuscitation period, hepatocyte isolation was carried out with nine livers. In eight livers, isolation was carried out without a resuscitation period. Enzyme concentrations are given in units per litre per 100 g liver weight, electrolyte concentrations in millimoles per litre, bile production in millilitres per 100 g liver weight per 3 h, and urea synthesis in milligrams per decilitre per 100 g liver weight per 3 h. The data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using Student's *t*-test.

Results

Normothermic perfusion period

K^+ The concentration of potassium decreased from 5 to 4.01 ± 0.16 mmol/l after 30 min and reached a concentration of 3.86 ± 0.09 mmol/l after 3 h of perfusion. During 3 h of perfusion the potassium concentration in the dialysate increased from 3.1 ± 0.15 prior to reperfusion to 3.82 ± 0.13 mmol/l after 180 min of perfusion.

Na^+ Sodium concentration decreased from 143 ± 1.89 mmol/l at the beginning of perfusion to 140.14 ± 1.45 mmol/l after 180 min. A steady state was reached

after 30 min of perfusion. The concentration of sodium in the dialysate at 0 and 180 min was 138 ± 1.96 and 139 ± 1.44 mmol/l, respectively.

pH PH of the perfusate was 7.42 ± 0.014 before reperfusion. After 30 min of perfusion a drop in pH to 7.28 ± 0.014 was measured. PH increased slightly thereafter. The pH measured was 7.35 ± 0.01, 7.37 ± 0.007 and 7.38 ± 0.005 after 60, 90 and 120 min, respectively. A steady state was reached after 120 min of perfusion. PH remained at 7.38 ± 0.003 until the end of perfusion. The pH of the dialysate at 0, 90 and 180 min was 7.47 ± 0.007, 7.37 ± 0.019 and 7.39 ± 0.01, respectively.

GOT The amount of GOT released after 30 min of perfusion was 59 ± 16 U/l per 100 g and increased to 85 ± 19 U/l per 100 g after 180 min. No GOT was detected in the dialysate.

GPT The largest amount of GPT was released during the first 30 min of perfusion. This concentration of GPT was 4.35 ± 1 U/l per 100 g and remained nearly constant to the end of perfusion. No GPT was detected in the dialysate.

LDH The amount of LDH released after 30 min of perfusion was 77 ± 18 U/l per 100 g. The concentration of LDH increased to 114 ± 19 U/l per 100 g after 180 min of perfusion. No LDH was detected in the dialysate.

Urea The livers produced on average 277 mg/100 g liver weight urea. This included the amount of urea dialysed.

Bile In all livers the gallbladder was drained and the cystic duct ligated. For that reason the bile collected during perfusion reflected the bile produced. Bile flow commenced after about 30 min of perfusion. After 180 min of perfusion 100 g liver produced on average 3.37 ± 0.6 ml bile.

Isolation

In the group with a 3-h resuscitation period, the mean viability as judged by trypan blue exclusion was $76.2 \pm 3.9\%$. The mean yield achieved was $72.8 \pm 1.5\%$ wet weight. The viability of hepatocytes isolated from livers without a resuscitation period after warm ischaemic injury was, on average, only $36.3 \pm 0.9\%$, with a mean yield of $54.4 \pm 1\%$ wet weight. The differences in viability and cell yield between both groups all reached statistical significance.

Phase contrast microscopic studies of the isolated cells were carried out for both groups. Viable cells showed a round morphology and a bright contrast between nucleus and cytoplasm. The plasma membrane showed a sharp refractile perimeter. Damaged cells were identified by addition of trypan blue, a swollen appearance, loss of contrast and discontinuities in the plasma membrane. Both groups differed in the amount of viable cells and in the degree of cell aggregation. In the resuscitation group, more intact cells were single units and the 38-h culture resulted in an even monolayer.

Discussion

By far the most hepatocyte isolation is carried out with organs that have not been subjected to warm ischaemia. Traditionally, the liver is thought to be an organ that is very sensitive to warm ischaemia. From more recent studies we know that the liver can tolerate warm ischaemic periods of over 60 min. [17]. Recent studies have shown that reperfusion of a liver after warm ischaemia ameliorates ischaemic injury. We showed that the ischaemic injury of livers subjected to 80 min of warm ischaemia was markedly reduced and their function improved by a normothermic perfusion over a period of 3 h. The perfusate contained HPF 3, whole blood, fructose, oleate and amino acids [11]. Integration of a dialysis circuit led to a further improvement as far as electrolyte homeostasis and pH were concerned [12]. It has been shown that regulation of cell metabolism is, in large part, dependent on cell volume [18], which is affected by electrolytes [19]. Haemodialysis helps in regulation of pH and electrolyte homeostasis and is a safe method for removing water soluble toxins and increased amino acids [20]. Our hypothesis was that if it was possible to reverse warm ischaemic injury by normothermic perfusion it might also be possible to achieve an improvement in hepatocyte isolation from ischaemically damaged livers.

We then investigated the possibility at isolating hepatocytes from resuscitated livers by an enzymatic "five-step" perfusion technique. Hepatocyte viability was, on average, 76%. The cell yield achieved was 73%. This was in accordance with data from the literature for hepatocyte isolation from livers without a preceding warm ischaemic period. Previous isolation of hepatocytes with the same modalities but without a 3-h resuscitation period resulted in far inferior results: viability was only 39% and the cell yield did not exceed 58%. We concluded that a 3-hour resuscitation period with improved HPF 3 and dialysis allows, for the first time, the isolation of hepatocytes in

large quantities with a high yield and an excellent viability. Our method allowed, for the first time, the utilization at abattoir livers for cell culture with good results, thus, saving laboratory animals, expense and hassle with administrative matters.

We believe that these results proved the concept of normothermic reperfusion for resuscitating livers. This concept might be of use in other parts of experimental surgery, such as for the isolation of islets cells from preperfused pancreas with an organ-specific modified regime. With further study and experimental work it might be possible to resuscitate human livers from non-heart-beating donors or from liver resections after partial hepatectomy. These livers might be of use for isolating human hepatocytes to be used in cultures and bioartificial liver support systems (bioreactor).

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