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OPTIMISATION OF PORCINE HEPATOCYTE CRYOPRESERVATION BY COMPARISON OF VIABILITY AND ENZYMATIC ACTIVITY OF FRESH AND CRYOPRESERVED CELLS

J. SMRŽOVÁ[a], Z. DVOŘÁK[c], J. LATA[a], P. DÍTĚ[a], M. MACHALA[b], L. BLÁHA[b], V. ŠIMÁNEK[c], J. ULRICHOVÁ[c]

[a]Department of Internal Medicine – Gastroenterology, University Hospital Brno, Czech Republic
[b]Veterinary Research Institute, Brno, Czech Republic
[c]Institute of Medical Chemistry and Biochemistry, Medical Faculty, Palacký University, Olomouc, Czech Republic

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Abstract

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Cryopreservation of porcine hepatocytes would ensure the accessibility of cells for laboratory use, permit the standardisation of experiments and save lives of animals. Therefore, in this study, we sought the optimal procedure for cryopreservation of porcine hepatocytes for both laboratory and clinical purposes.

Hepatocytes were isolated from the liver lobe of a mini-pig by two-step collagenase perfusion. The cells were frozen with 20% foetal calf serum and 15% DMSO in two different media in four different concentrations ranging from 1×10^6 cells/ml to 5×10^6 cells/ml. For this purpose, 1.8 ml cryotubes and 120 ml Baxter bags were used. Cells were cryopreserved either in a controlled freezer Sylab or step by step in a styro-foam box and stored at -196 °C.

The quality of fresh and cryopreserved hepatocytes was assessed by trypan blue exclusion test and by the evaluation of cytochrome P450 isoenzymes and glutathione-S-transferase activities; primary cultures were evaluated morphologically and by MTT test. Cryopreserved hepatocytes did not form the typical monolayer of polygonal cells in primary cultures and remained round, unlike fresh hepatocytes. Lifetime of viable culture was shortened from 7-8 days to 4 days in cryopreserved cells. Viability of fresh cells was 88 ± 2% and decreased to 36-63% in cryopreserved hepatocytes. Enzyme activities of cryopreserved cells were reduced to 60% when compared with fresh hepatocytes. Concentrations of 3×10^6 cells/ml and 5×10^6 cells/ml and controlled freezing gave the best results. The use of Baxter bags was more convenient due to easier manipulation. Freezing media appeared to have no influence.

Freezing conditions, MTT test, cytochrome P450, primary cultures

Hepatocytes are being increasingly employed for basic and pre-clinical research and their clinical use has also become more common in recent years. Clinical experiments, phase I-II with bioartificial liver (bioreactor) have been performed with porcine hepatocytes (Watanabe 1997) or immortalised human cell lines (Sussman 1994), and the first reports on human hepatocyte transplantation in the case of inherited metabolic disorders (Fox 1998) and in fulminant liver failure (Habibullah 1994) have appeared. However, the availability of either animal or human hepatocytes in particular has not improved comparably. Fewer difficulties are met in obtaining animal hepatocytes but these are often not suitable for clinical use or not usable in some laboratory tests for different reasons (immunological, functional, ethical, infectious, legal, etc.).

Hepatocytes do not proliferate in primary cultures without addition of specific growing factors and this is why every cell we need has to be obtained from the liver. While the quantity of cells that can be yielded from one liver specimen is very large (Kosina 1999), relatively few

Address for correspondence:
MUDr. Jana Smržová
Dept. of Internal Medicine-Gastroenterology
Univestity Hospital Brno
Jihlavská 20, 639 00 Brno, Czech Republic

Phone: +420 5 4719 3828
Fax: +420 5 4719 3701
E-mail:jsmrzova@fnbrno.cz
http://www.vfu.cz/acta-vet/actavet.htm

cells are needed for experiments. These facts have led researchers all over the world to seek a procedure that would permit hepatocyte preservation. For a shorter period, cold preservation is quite appropriate but for longer time periods, it is necessary to cryopreserve the cells.

Cryopreservation of hepatocytes offers a wide range of advantages. Different experiments can be carried out on cryopreserved hepatocytes from one source, or the same experiment can be consecutively repeated on the same cells what obviates inter-individual variability. On the other hand, inter-individual differences can be studied in a single experiment. The same cells can be used in different laboratories as well. The latter offers the possibility of advantageous experimental standardisation. Complete utilisation of isolated hepatocytes enables us to reduce the number of slaughtered animals (De Sousa 1999). In addition, experience in animal hepatocyte cryopreservation can be used in the case of human hepatocytes (Hengstler 2000). Cryopreservation of human hepatocytes allows us to work on cells with limited availability (Dvořák 2000; 1999). Cryopreserved hepatocytes can also be used for ex vivo experiments: biotransformation of xenobiotics (Li 1999; Salmon 1996; Swales 1998; Zaleski 1993), enzyme induction (Madan 1999; Jamal 2000), drug-drug interaction (Li 1997, 1999; Olsen 1997) and hepatotoxicity (Li 1999). Their clinical use, however, requires better metabolic characterisation and an optimal cryopreservation procedure in order to improve cell viability and metabolic activity after thawing (Li 1999); enzyme induction studies are needed as well (Madan 1998).

Various modifications of cryopreservation conditions including different media, cell concentration, cytoprotective agents etc. have been already described (Naik 1997; Chesné 1993; Guillouzo 1999; Dvořák 2000; De Loecker 1998). There are substantial differences in freezing and defrosting curves as well as in pre- and post-freezing procedures. It is difficult to compare the results because of the large number of frequent minor modifications and different methods used in cryopreserved cell quality evaluation. Comparative studies are still missing.

The aim of our study was to provide practical instructions for hepatocyte storage by cryopreservation and to compare the quality of fresh and cryopreserved cells. From a wide range of methods that are used to assess hepatocyte viability we chose for this study the trypan blue exclusion test, microscopic evaluation of primary cultures and MTT test carried out at different time points. Metabolic activity was monitored by cytochrome P450 isoenzymes (CYP) - 7-ethoxyresorufin-O-deethylase (EROD) that is specific for CYP 1A1/CYP 1A2, 7-pentoxyresorufin-O-deethylase (PROD) specific for CYP 2B and 7-benzyloxyresorufin-O-demethylase (BROD) that shows the activities of CYP 3A/ CYP 2B. Glutathione-S-transferase was taken as a marker of phase II biotransformation.

Materials and Methods

Materials

Experimental mini-pigs were purchased from the Institute of Animal Physiology and Genetics, Liběchov, Czech Republic.

Collagenase cruda was purchased from Sevac (Czech Republic) and trypan blue from Merck. Hepatocyte Medium (HM) and all other chemicals were from Sigma. All chemicals were of a quality suitable for tissue cultures.

The composition of media (Modrianský 2000):

HEPES 1 consisted of HEPES (20 $mmol \cdot l^{-1}$), NaCl (120 $mmol \cdot l^{-1}$), KCl (5 $mmol \cdot l^{-1}$), glucose (28 $mmol \cdot l^{-1}$), mannitol (100 $\mu mol \cdot l^{-1}$), sorbitol (100 $\mu mol \cdot l^{-1}$), glutathione (100 $\mu mol \cdot l^{-1}$), penicillin G (10 U/ml), streptomycin (100 $\mu mol \cdot l^{-1}$), and amphotericin B (25 $mg \cdot l^{-1}$), pH 7.4. HEPES 2 consisted of HEPES (20 $mmol \cdot l^{-1}$), NaCl (120 $mmol \cdot l^{-1}$), KCl (5 $mmol \cdot l^{-1}$), glucose (28 $mmol \cdot l^{-1}$), penicillin G (10 U/ml), streptomycin (100 $\mu mol \cdot l^{-1}$), and amphotericin B (25 $mg \cdot l^{-1}$), pH 7.4. HEPES 3 had the same composition as HEPES 2 plus $CaCl_2$ (0.7 $mmol \cdot l^{-1}$), and 500 mg collagenase (530 U/g). HEPES 4 had the same composition as HEPES 2 plus 1% foetal calf serum. EGTA medium consisted of EGTA (0.5 $mmol \cdot l^{-1}$), KCl (5.4 $mmol \cdot l^{-1}$), KH_2PO_4 (0.44 $mmol \cdot l^{-1}$), NaCl (140 $mmol \cdot l^{-1}$), Na_2PO_4 (0.34 $mmol \cdot l^{-1}$), and Tricin (25 $mmol \cdot l^{-1}$), pH 7.2. Culture medium (LHM) consisted of Willliams' medium E and HAM F12 in a 1:1 ratio including the following additives: glucose (7 $mmol \cdot l^{-1}$), glutamine (2.4 $mmol \cdot l^{-1}$), penicillin (100 U/ml), streptomycin (10 $\mu mol \cdot l^{-1}$), sodium pyruvate (0.4 $mol \cdot l^{-1}$),

dexamethasone (1.8 μmol·l^{-1}), holo-transferrin (5 mg·l^{-1}), ethanolamine (1 μmol·l^{1}), insulin (350 nmol·l^{-1}), glucagon (0.2 mg·l^{-1}), linolic acid (11 μg·l^{-1}), and amphotericin B (1.4 mg·l^{-1}), pH 7.2 (Isom 1985).

Methods

Hepatocyte isolation

Porcine hepatocytes were obtained from the liver lobe of a freshly slaughtered experimental mini-pig (body mass 25 kg) using two-step collagenase perfusion (Modriansky 2000). Following the usual slaughtering process, the liver was excised by butchers and transported rapidly in a plastic bag in ice water to the laboratory. The liver lobe weighing 200-250 g was placed on a perforated board in a sterile dish and perfused with 1000 ml of the HEPES 1 solution with the aid of a catheter placed in the natural orifices on the cut face of the liver. Regular flow of 100 ml/min was maintained by a peristaltic pump. The liver was then perfused with 1000 ml of EGTA solution and followed by 1000 ml of HEPES 2 solution. After removing all the liquid from the dish, the HEPES 3 solution containing collagenase and Ca^{++} ions was recirculated for about 10 min until the surface of the lobe was soft. The liver lobe was then transferred into 100 ml of cold (4 °C) HEPES 4 solution and mashed using scissors. The cell suspension was diluted in a HEPES 4 solution to 400-600 ml and filtered through sterile gauze. The cells were washed three times in the LHM culture medium. Centrifugation for 3 min at 50 × *g* at room temperature followed each wash.

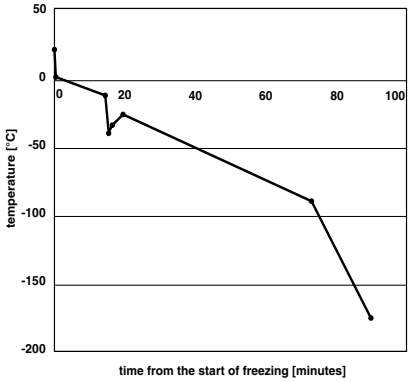

Fig. 1. Freezing curve in the controlled freezer Sylab

Freezing of hepatocytes

Hepatocytes were cryopreserved in 1.8 ml cryotubes or in 120 ml Baxter bags. The hepatocyte suspension was diluted in freezing medium (LHM or HM) supplemented with foetal calf serum. For each cryotube, 0.9 ml of the hepatocyte suspension was mixed slowly drop by drop to avoid osmotic shock with 0.9 ml of ice-cold solution of DMSO and Hank's buffer. Final concentrations were: DMSO 15%, Hank's buffer 35%, foetal calf serum 20% and hepatocytes in freezing medium 30%, cell concentrations 1, 2, 3 and 5 × 10^6 cells/ml. Cryotubes were next immediately transferred into the freezer Sylab. Controlled freezing up to -196 °C was performed for 90 min with cooling rate adjusted for the heat of crystallisation (see Fig. 1).

For comparison, slow freezing in a styro-foam box was performed by placing the box into -80 °C with the transfer of cryotubes into -196 °C after 24 h.

Storage

Cryopreserved hepatocytes were stored in liquid nitrogen at –196 °C for 3 months.

Thawing

Cryotubes were thawed separately in order to reduce the time of contact of defrosted cells with toxic DMSO. Each cryotube was placed into a 37 °C warm bath and in the course of thawing, the suspension was transferred step by step into a cultivation medium (for primary cultures) or buffer solution PBS (for enzyme activities testing) of threefold to fourfold volume. After 10 min of incubation, the suspension was centrifuged 3-4 min at 50 × *g* at room temperature and the supernatant was removed and replaced by fresh medium or buffer solution, respectively. The cell viability was then determined.

Trypan blue exclusion test

The viability of hepatocytes was determined using the trypan blue exclusion test. In this test, 10 μl of cell suspension was added to 1 ml of 0.5% trypan blue in PBS. The number of dead (blue) and living (white) cells was counted in a Bürker chamber.

Primary cultures

Hepatocytes were seeded on rat tail collagen type I-coated Petri dishes or 6-well plastic dishes at final cell concentration 1.25 × 10^5 cells/cm^2. For comparison, non-coated Petri dishes were employed. To allow cell attachment and formation of monolayer, culture medium (LHM or HM) supplemented with 5% foetal calf serum was initially used. The culture medium was exchanged for a serum-free one after a four-hour incubation. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT test

MTT test was performed immediately after isolation, or thawing. After exchange of culture medium, 100 μl of MTT solution (5 mg/1 ml PBS) was added to primary culture. 3 h later, the medium was removed and 1 ml DMSO + 1% ammonia was added. After 5 min of incubation, absorbance at 540 nm was measured (UV VIS reader Anthos, HT2, type 12500).

Enzyme activities

Activities of 7-ethoxyresorufin-O-deethylase (EROD), 7-pentoxyresorufin-O-deethylase (PROD) and 7-benzyloxyresorufin-O-demethylase (BROD) were determined fluorimetrically according to Prough (1978) at 30 °C. Activity of glutathione-S-transferase was measured spectrophotometrically according to Habig (1974) with 1-chloro-2,4-dinitrobenzene (1.25 mM) as a substrate.

Data represent the mean ± SD of 2-3 independent experiments determined in duplicate.

Results and Discussion

The viability of *freshly isolated hepatocytes* was 88 ± 2%. Fresh hepatocytes formed the typical monolayer of polygonal cells from the second day of cultivation. The morphologic appearance of the culture remained stable for 6-7 days when the hepatocytes became round and seceded from the Petri dish surface on day 7-8 of the culture. During this stable period, we observed no deterioration of cell nucleus, cytoplasm or membrane. Hepatocytes showed satisfactory metabolic activity just after the isolation: EROD 6.4 pmol/min/10^6 cells, PROD 0.95 pmol/min/10^6 cells, BROD 0.19 pmol/min/10^6 cells, GST 0.322 mmol/min/10^6 cells. Increased metabolic activity as MTT test rate at 20 and 40 h (see Fig. 3) proves adaptability of hepatocytes to cultivation conditions and the cells' suitability for experiments after 24 h of stabilisation.

Concerning hepatocyte viability, our results are comparable to other studies where hepatocytes were isolated from separate liver lobes (Guillouzo 1999; Chesné 1993).

The hepatocyte cryopreservation and tests listed above were carried out with the following modifications. We compared two *different media* for hepatocyte cryopreservation, the Hepatocyte Medium Sigma and LHM culture medium. We failed to demonstrate any difference between these media using the trypan blue exclusion test, MTT test and enzyme activities (data not shown). There are several reasons to explain this fact. The difference in composition of tested media was too small, or cryoprotective agents and cryopreservation procedure have much greater impact on the result than the medium used.

Two *cryopreservation methods*, the cryopreservation in a styro-foam box and controlled freezing were compared. We found no significant difference between these methods in regard to cell viability in the trypan blue exclusion test (see Fig. 2), but there was a significant difference in MTT test results at 20 h and 40 h, where controlled freezing gave much better results (see Fig. 3). At 3 h, the box freezing seemed to be superior to controlled freezing, but we must take into consideration the fact that there may be some remaining enzyme activities released from damaged cells and therefore the results at 20 or 40 h are of greater importance. There were no important differences in enzyme activities (EROD, PROD, BROD, GST)

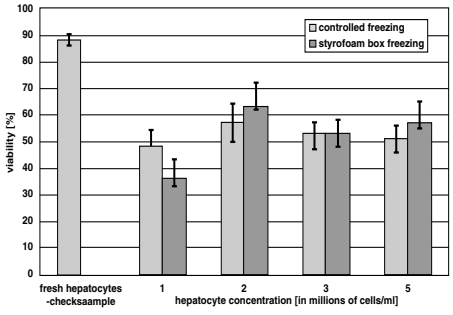

Fig. 2. Hepatocyte viability in trypan blue exclusion test and its dependence on cell concentration and cryopreservation method (controlled freezing vs. styrofoam box freezing), cultivation on collagen-coated Petri dishes in Hepatocyte medium Sigma

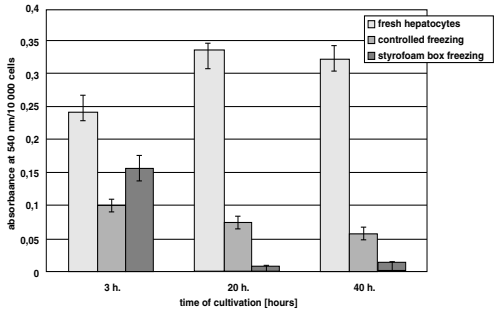

Fig. 3. Mitochondrial activity according to the MTT test in fresh and cryopreserved cells (controlled freezing vs. box freezing) at 3, 20 and 40 h, freezing cell concentrtion 3 mil. cells/ml, cultivation on collagen-coated Petri dishes, Hepatocyte medium

either between fresh and cryopreserved hepatocytes or between box-frozen and controlled-frozen cells. However, these tests were carried out just after isolation and thawing and therefore, released enzyme activities may have modified the results. It is desirable to perform these tests over time. If we consider the best predictive value of the MTT test, we conclude that controlled freezing is more convenient for hepatocyte cryopreservation.

The optimal *cell concentration* in the hepatocyte suspension during freezing was also evaluated. According to the literature, this can influence cell cryopreservation, but the optimal cell concentration has not yet been determined. In the specimen from the first mini-pig, quite good results were obtained for cell viability, MTT test and enzyme activities in concentrations 2×10^6 cells/ml, 3×10^6 cells/ml and 5×10^6 cells/ml, but concentration of 3×10^6 cells/ml gave the best results. In the other specimen, the cell concentrations of 3×10^6 cells/ml and 5×10^6 cells/ml were superior to the others. The concentration of 1×10^6 cells/ml turned out to be the least valuable (see Fig. 2). The explanation for this phenomenon may be the presence of intercellular interactions that protect cells against freezing stress. This may be important even during the short period of freezing and defrosting. We considered the influence of quantity of cryoprotective agent per cell depending on cell concentration too, but this should not be influential because of the relatively small cell concentration used. In future experiments, we plan to test higher cell concentrations for economic reasons.

For comparison, we cultivated hepatocytes both on *collagen-coated and non-coated Petri dishes*. Surprisingly, there was no difference in the morphologic appearance or in the lifetime of the cultures. On the other hand, a significant difference between coated and non-coated dishes was found in MTT test (data not shown). For every cell concentration at all time intervals, coated dishes gave better results. This correlates with the literature and demonstrates that collagen coating enhances the metabolic activity of mitochondria. For this reason, we performed all further experiments on coated dishes.

For simpler manipulation, 120 ml *Baxter bags* were tested because one bag contains the same quantity of hepatocytes as more than 60 cryotubes. These bags are routinely used for bone marrow or peripheral blood stem cells cryopreservation. We employed the same cryopreservation procedure as for cryotubes. Preliminary experiments showed comparable viability of cryopreserved hepatocytes.

For further discussion, we selected data from the tests with the most appropriate conditions (according to our results as shown above), i.e. cryopreservation of hepatocytes in cryotubes in the concentration of 3×10^6cells/ml, by controlled freezing and with HM as freezing medium.

After cryopreservation, the viability of hepatocytes dropped to 36-63% (see Fig. 2) according to the cell concentration, and was 53% for the concentration of 3×10^6 cells/ml. In primary cultures, the cell morphology was modified, the hepatocytes remained round during the whole culture lifetime, with only a very small number of cells forming the typical polygonal shape. For this reason we assume that the cells did not form intercellular contacts as they do in primary cultures of fresh hepatocytes (electron microscopy could elucidate this question). The lifetime of primary cultures was shortened to 4 days. The decrease in mitochondrial activity after 3 h of incubation corresponded to drop in viability of cryopreserved cells, and there was a further fall at 20 h and 40 h, not observed in fresh hepatocytes. By contrast, fresh hepatocytes gave better results at 20 h and 40 h, probably due to the cell reparation in the primary culture after the initial isolation injury that does not occur in cryopreserved cells (see Fig. 3). Cytochrome P450 isoenzymes (EROD, PROD, BROD) and glutathione-S-transferase activities were quite well conserved in cryopreserved cells, reaching more than 60% of initial values for all cell concentrations used (see Fig. 4, data selected for cell concentration 3×10^6 cells/ml). However, these activities were

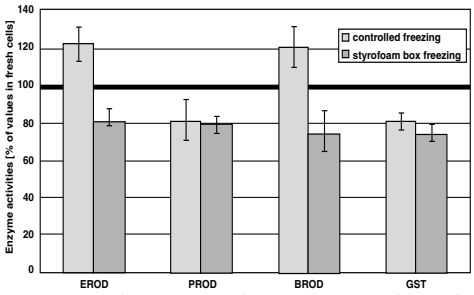

Fig. 4. Enzyme activities of cryopreserved hepatocytes after thawing (controlled freezing vs. styrofoam box freezing) in comparison to fresh cells (100 %), freezing cell concentration 3 mil. cells/ml, freezing medium Hepatocyte medium Sigma

examined only immediately after the thawing and therefore their important part may be due to enzymes released from damaged cells. Their decrease over time can be expected, according to the result of the MTT test, and thus cryopreserved hepatocytes are not ideal for primary cultures. By contrast, they are suitable for metabolic studies owing to their well-preserved enzyme activities after thawing.

Conclusion

The results of this study demonstrate the need for further procedural refinement. This cryopreservation procedure allows us to start short-term experiments with cryopreserved cells in suspension with all the advantages described above. However, we need to improve the freezing procedure in order to obtain cells of the quality permitting cryopreserved hepatocyte cultivation in primary cultures. In addition, optimisation of cultivation conditions will be the subject for further studies.

Optimalizace kryoprezervace prasečích hepatocytů srovnáním životnosti a enzymatické aktivity čerstvých a kryoprezervovaných buněk

Kryoprezervace prasečích hepatocytů by zabezpečila dostupnost buněk pro laboratorní užití, dovolila standardizaci experimentů a ušetřila životy zvířat. V této práci jsme proto hledali nejlepší postup kryoprezervace prasečích hepatocytů jak pro laboratorní, tak pro klinické účely.

Hepatocyty byly izolovány z jaterního laloku experimentálního miniaturního prasete pomocí dvoustupňové perfúze kolagenázou. Buňky byly poté zamraženy ve 20% fetálním telecím séru a 15% DMSO ve 2 různých médiích ve 4 koncentracích od 1×10^6 buněk/ml do 5×10^6 buněk/ml. Použity byly kryotuby o objemu 1.8 ml a vaky Baxter o objemu 120 ml. Buňky byly zamraženy buď řízeným mražením v přístroji Sylab, nebo postupným ochlazováním v polystyrénových boxech a poté uchovávány při -196 °C.

Kvalita čerstvých a kryoprezervovaných hepatocytů byla hodnocena v testu s trypanovou modří a měřením aktivity izoenzymů cytochromu P450 a glutathion-S-transferázy; primární kultury byly posuzovány morfologicky a testem MTT. Kryoprezervované hepatocyty nevytvářely v primárních kulturách typickou monovrstvu polygonálních buněk a zůstávaly na rozdíl od čerstvých hepatocytů okrouhlé. Životnost primárních kultur kryoprezervovaných buněk byla zkrácena ze 7-8 dní na 4 dny. Viabilita čerstvých hepatocytů byla $88 \pm 2\%$ a poklesla u kryoprezervovaných buněk na 36-63%. Enzymatická aktivita kryoprezervovaných buněk činila přes 60% hodnot naměřených u čerstvých hepatocytů. Nejlepší výsledky byly při použití koncentrací 3×10^6 buněk/ml a 5×10^6 buněk/ml při řízeném mražení. Použití vaků Baxter nabízelo výhodu snazší manipulace. Neprokázali jsme vliv použitého zamražovacího média.

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