Journal of Clinical & Experimental Pathology

Li et al., J Clin Exp Pathol 2017, 7:5
DOI: 10.4172/2161-0681.1000322

Review Open Access

Isolations and Cultures of Primary Hepatocytes

Yi Li[1,2], **Mengyu Gao**[1,2], **Diwei Wu**[1,2] **and Ji Bao**[1,2*]

[1]*Laboratory of Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China*

[2]*Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu 610041, China*

[*]**Corresponding author:** Ji Bao, Laboratory of Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China, E-mail: baoji@scu.edu.cn

Received date: September 18, 2017; **Accepted date:** September 22, 2017; **Published date:** September 25, 2017

Abstract

Various liver diseases result in liver failure, and liver transplantation and artificial liver support systems are emerging as alternative therapies for these disease. However, both treatments are depending on cell resources, so isolating sufficient number of functional primary hepatocytes is a most important early issue. Isolated hepatocytes are also a suitable system for the physiological, pharmacological and toxicological study of hepatic uptake, metabolism, excretion and toxicity. In this paper, the methods for hepatocyte isolation are reviewed; the culture and assessment methods are also examined. Hepatocyte transplantation and hepatocyte-based bioartificial liver support systems have attracted the attention of researchers. From a future perspective, developing of gene engineering is emerging as a promising way to modify cells, and this is renewing interest in the development of methods for isolation and culture of hepatocytes.

Keywords: Liver transplantation; Liver cirrhosis; Hepatocytes

Introduction

The liver is the largest internal organ in the body, and performs a complex and indispensable array of more than 500 functions including immunologic complements and coagulation factors synthesis, and the metabolism of nutrition, drugs and detoxification toxin substance [1]. Viral hepatitis, fatty liver disease, drug-induced liver injury, liver cirrhosis, hepatic carcinoma, and other liver diseases can cause liver failure [2]. Orthotopic liver transplantation is the only proven therapy for liver failure, but its overall usage is limited by the need for major surgery, life-long immunosuppression, and the shortage of donor organs [3].

Freshly isolated primary hepatocytes retain their liver-specific functions [4]; therefore, they continue to be a valuable cell resource to develop alternative treatments such as hepatocyte transplantation and bioartificial liver support systems for the treatment of acute liver failure, chronic liver diseases and several inherited metabolic disorders of the liver. In addition, primary hepatocytes have been used for many aspects of biochemical and pharmaceutical research to understand the biological processes occurring in the liver.

Primary hepatocytes were first isolated from rats [5]. They were later isolated from many other species, including mice [6], bovines [7], pigs [4,8] and human [9]. Large-scale of hepatocytes without the loss of essential specific differentiated functions are required to advance above-mentioned tissue engineering, gene therapy and extracorporeal liver support systems [10,11]. Thus, every step toward a gentler method of isolating primary hepatocytes to increase their number, vitality, and quality is critical to develop and worthy of thorough review. The purpose of this mini review covers the current status of hepatocyte isolations and cultures, and describes some techniques used to assess of hepatocyte function, in view of a potential laboratorial and clinical use.

Hepatocyte Isolation

Hepatocytes are by far the most prominent morphological component of the liver, accounting for about 78% of liver volume and more than 65% of cell number [12]. Non-parenchymal cells such as endothelial cells and Kupffer cells, stellate cells are also resident in the liver [13]. The liver parenchyma is permeated by a collagenous fibrillar network, which is embedded in an extracellular matrix, mainly composed of a mixture of elastin, heparin sulfate proteoglycan and surface adhesion molecules, such as laminin and fibronectin [14]. The extracellular matrix, adhesion glycoproteins, Ca^{2+} ions and cell surface receptors play an important role in maintaining cell anchorage, shape, polarity and function [15]. Variations of extracellular matrix content between different species result in the degree of difficulty. For example, the porcine liver contains more collagen and fibrous tissue, thus it is more difficult to dissociate than the rat liver.

Mechanical dissociation of the hepatocytes has been replaced by the enzymatic digestion, because the former is more aggressive to the liver parenchymal. Perfusion of the liver with collagenase showed higher cell yield than immersion of the chopped liver in collagenase solution. Whole liver organs or resected liver tissues are both available to isolate hepatocytes, as long as they fulfill the criteria regarding the absence of infectious agents or high level of hepatic lesions. Various routes can be exploited to administer hepatocytes in whole liver organs: intrasplenic, directly into the hepatic parenchyma, or in the peritoneal cavity, but more often intraportal [16]. Left lateral sector segments are usually suitable to obtain normal resected tissues, with proportionate volumes, after which the lobular blood vessels can be easily exposed for catheterization. It is important that the liver lobe should be incised with a single cut, and then the lobular blood vessels can be exposed for catheterization.

Enzymatic perfusion method is based on the permeation of the liver with Ca^{2+} chelator solutions to disrupt Ca^{2+}-dependent (cadherin-based) cell junctions, and enzymatic digestion of the liver parenchyma to disrupt cellular-EMC interactions [17]. The enzyme perfusion time required for digestion is not pre-determined; rather, perfusion

continues until adequate tissue digestion is achieved. The enzyme is temperature sensitive; the activity of the collagenase can be greatly influenced, leading to the decrease of the viability in isolated hepatocytes. The optimum temperature of enzyme solution is 37-38°C; this temperature is measured from the perfusion site (liver lobes) rather than solution container. The temperature of the solution ought to be controlled all the time based on this temperature to obtain the optimal efficiency. Besides, the solutions usually perfused hollow-fiber bioreactor to enrich O_2, to reduce the damages of the isolated hepatocytes.

Collagenase type IV was a classical and broad spectrum enzyme used in primary hepatocytes isolation in all species. With upgrade of commercial collagenase products such as Roche Liberase research grades and Serva collagenase NB grades, the isolations are more specific to the species, while the purity, viability, and yield of isolated primary hepatocytes are highly improved [4,18]. Except for the primary component, highly purified collagenaseand Ⅱ mixture, we found that the amount of neutral protease thermolysin also determines the viability and cell yield, and especially the further success of spheroid formation. If the thermolysin is too much, the spheroids usually are not round in shape; cells in them attach with each other, rather than aggregating, and are mostly covered by the blebs. N-acetylcysteine (NAC) is an antioxidant that acts through the replenishment of glutathione in the liver. It also has direct antioxidant properties and appears to have hepatoprotective effects against liver ischemia/reperfusion injury to improve the viability [9]. It has been proved to improve the viability and conserves the metabolic function of hepatocytes [4,18].

Iced medium containing Williams'-E can terminate enzyme digesting, help to maintain the cell activity, and remove the damage cells. Mechanical dissociation and filtering is necessary to remove connective tissue, and subsequent centrifugation is required to separate hepatocytes from both dead hepatocytes and non-parenchymal cells [4]. Subsequent centrifugation can enrich hepatocytes, and remove part of contamination of other cell types, but when the yield is low, or when selective removal of all non-parenchymal cells, purification is required. Percoll or Ficoll solutions have been developed for purifying suspensions of hepaotcytes [19]. The discrepancy of buoyant densities between hepatocytes and non-parenchymal cells facilitates the process of cell separation. Washing and centrifuging are important, because if dead hepatocytes are not removed immediately, they will release large amounts of DNA, lactate dehydrogenase, and intracellular antigens, thus further reducing cell viability.

Three quick tests were studied to evaluate the success of single hepatocyte isolation, by assessing cell yield, viability and morphology. The cell yield is usually calculated by the dry weight of hepatocyte mass. The most commonly used method to assess hepatocyte viability is the trypan blue exclusion test. To ensure persistent good viability during culture, at least 90% of the cells should exclude trypan blue on first examination. Light microscopy examination of the hepatocytes provides information on the quality of cells. Blebbing of the plasma membrane is a sign of fluid uptake by damaged or over digested hepatocytes, which is often followed by cell death [20]. The transmission electron microscopy (TEM) examination is not usually necessary in each isolation. On TEM, injury of plasma membrane, swelling of mitochondria, degranulation of endoplasmic reticulum and vesiculation of the Golgi apparatus, as well as vacuolization of the cytoplasm are frequently seen in damaged or over digested hepatocytes cells [21].

Hepatocyte Culture

Primary hepatocytes do not proliferate *in vitro* and therefore need to be freshly isolated for each experiment [22]. Once plated in a monolayer, primary hepatocytes typically undergo progressive dedifferentiation within 72 h *in vitro*, which is reflected at the level of the drug transporters and the dramatic loss in the phenotypic characteristics of the cells.

Conventional approaches to counteract this dedifferentiation aim at reestablishing the natural hepatocyte microenvironment by introducing extracellular matrix [23]. One common technique is to sandwich the cells between layers of collagen gel [24] or matrigel [25]. Unfortunately, sandwich cultures do not provide the complex multi-cellular environment found *in vivo*, and co-cultures do not mimic the layered liver architecture. The liver-specific functions in this method decline within the first week, suggesting that significant survival factors are missing [26]. Other current techniques that maintain hepatocellular function *in vitro* with different biomaterials and geometries exhibit a relatively low cell density and functional capacity per unit volume, and still demonstrate the limitation to reproduce hepatocytes *in vitro* [25].

Isolated hepatocytes can also be cultured in the medium in the form of suspended spheroids. Addition of differentiation promoting soluble compounds to the culture medium can boost the establishment of homotypic hepatocyte interactions. For example, EGF and HGF were demonstrated to induce DNA synthesis in primary hepatocytes [27]. These kinds of essential factors were determined and supplement in the medium for keeping liver cells vigorous. Moreover, co-culture with other cell types may be responsible for re-establishing cell junctions such as E-cadherin which is required for hepatocyte spheroid formation [28], and protecting hepatocytes from cell death, thus is emerging as a prospective way to cultivate and maintain hepatocytes. Seeding the primary hepatocytes with mesenchymal stem cells [29], liver sinusoidal endothelial cells or umbilical vein endothelial cells [30] has been reported to result in enhanced heterotypic cell-cell interactions to form spheroids in a shorter time, which led to improvements in hepatocyte function and prolongation of the survival time. Cells in these aggregates maintain their morphology and liver specific functions for over one month.

Hepatocyte spheroids can be maintained in serum-free and serum-containing culture medium. Serum-containing formulations are often based on Williams'-E Medium and are ideal for short-term cultures, up to 10 days. Serum-free formulations were developed for long-term cultures (several weeks) and require an adaptation period [29].

A batch of tests can be used to evaluate the ability of hepatocytes to survive, metabolize and excrete. First of all, hepatocytes contain numerous mitochondria in their cytoplasm, and show active cellular respiration. A steady decline in oxygen consumption indicates progressive cell damage. Second, albumin synthesis is one of classical assays for the study of liver-specific function. Monolayer-cultured primary hepatocytes rapidly lose albumin secretion *in vitro*, while self-assembled hepatocyte spheroids can keep alive and maintain high level of albumin secretion for over 50 days in continuous cultivation. However, we found that quantification of albumin is not consistent with the hepatocyte functions.

The function of isolated hepatocytes is more critical to be measured by assessing of ammonia degradation and ureagenesis. The study is performed after addition of 1 mM final concentration of ammonium such as NH4Cl into the medium. In some continuous culture circumstance, even with high level of albumin production, the other functions like ureagenesis or cytochrome P450 would drop obviously. However, while hepatocytes demonstrate a higher ammonia clearance rate, the diazepam metabolism is more active, showed in our unpublished preliminary studies.

When come to clinical use in the future, commercial primary hepatocytes products will be available. Minimal criteria for defining release specifications should be established and standardized. Such criteria should not be confused with identifying criteria for research purposes. Clinical doctors should reach an agreement on the criteria to decide the usability of the hepatocytes.

Perspectives

New clinical perspectives of allogenic and xenogenic isolated hepatocyte utilization have recently been proposed, primarily for hepatocyte transplantation and as the basis of liver support systems to replace compromised liver function.

For hepatocyte transplantation, isolated liver cells have been used in a variety of configurations: suspended, matrix-attached and encapsulated, singularly or in small aggregates. Transplantation of isolated xenogenic hepatocytes into the peritoneal cavity, into the spleen or directly injected into the liver *via* the portal vein has been performed for the treatment of experimental acute liver failure [31]. Transplanted hepatocytes were shown to survive and function throughout the life-span of recipient small experimental animals. Several inherited metabolic disorders of the liver such as Nagasean albuminemic in naturally mutant animals have also been treated successfully by hepatocyte transplantation [32].

Isolated primary hepatocytes can be used to construct bioartificial liver support systems to treat patients with severe acute failure [10]. The rationale of such systems is to bridge the severely ill patient to the liver transplantation or recovery by supplement them with the essential liver functions. It has been assumed that 10-15 $\times$ 10^9 cells need to be replaced to support an acutely failing liver in adults [33]. Such large scale of hepatocytes is difficult to acquire, and the liver-specific function is another critical problem. The metabolic profiles of porcine hepatocytes are basically similar to those of humans, and freshly isolated primary hepatocytes retain most of their liver-specific functions; therefore, porcine hepatocytes are a reasonable alternative to human hepatocytes. Once a standardized method of isolating primary hepatocytes has been established, isolated liver cells will be easily accessible and more frequently available. One of the most important early issues has been solved in the initial approach to the construction of bioartificial liver devices.

Genetically altered hepatocyte use could represent the first choice for treatment of specific genetic defects of liver function. Defects of some genes, which are preferentially expressed in liver cells, can cause metabolic disorders and inherit to next generations. For example, hereditary tyrosinemia type 1 (HT_1) is an autosomal recessive inborn error of metabolism, caused by deficiency in fumarylacetoacetate hydrolase (FAH), an enzyme that catalyzes the last step of tyrosine metabolism [34]. Absence of FAH causes accumulation of fumarylacetoacetate, resulting in mutagenic, cytostatic, and acutely apoptotic events within the cell [35]. Hypothesis that normal copies of FAH genes can be introduced into isolated hepatocytes from partial liver resection is proposed. Indeed, animals showed corrected cells proliferated and functioned after they received autologous transplants of hepatocyte underwent gene therapy [36].

Bioengineered FAH-deficient pigs can also serve another use as *in vivo* incubators for the large-scale production of primary human hepatocytes. It is based on the notion that the administration of 2-(2-nitro-4-trifluoromethylbenzyol)-1,3cyclohexanedione (NTBC), a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase, can control the progress of HT1 [37]. When the animals are injected of allogeneic normal cells, or ideally, xenogeneic cells from human, and gradually withdrawn NTBC, FAH-deficient cells selectively undergo severe dysfunction, most frequently leading to death [38]. Even though some of the preliminary researches have proved that there is no immunoreactivity when patients were treated with xenogenic cells, if appropriate hollow-fiber was employed, human hepatocytes are still the preferred source of cells.

Conclusions

Hepatocyte isolation started by Berry and Friend in the mid-1960s [5]. After the first isolation, many innovative techniques were introduced to refine the method. The modified methods, compared to the traditional methods, can improve results, allowing isolation of a large number of hepatocytes with high quality. Hepatocyte isolation is a time-consuming and costly procedure. Every factor including the tissue weight, liver lesion such as steatosis or cirrhosis, and blood supply status of surgical specimens can affect the cell yield and viability of isolated hepatocytes. Hepatocyte-like cells derived from induced pluripotent cells and cell lines like C3A or $HepG_2$ demonstrate some safety concerns and insufficient metabolism capability. Hepatocyte transplantation and hepatocyte-based bioartificial liver support systems have attracted the attention of researchers in the field, and developing of gene engineering is emerging as a promising way to modify hepatocytes. This is renewing interest in the development of methods for isolation and culture of hepatocytes.

Acknowledgements

This study was supported by the National Natural Scientific Foundations of China (no. 81770618).

References

1. Shulman M, Nahmias Y (2013) Long-term culture and co culture of primary rat and human hepatocytes. Methods Mol Biol 945: 287-302.
2. Hu C, Li L (2015) In vitro culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. Protein Cell 6: 562-574.
3. Brown RS (2008) Live donors in liver transplantation. Gastroenterology 134: 1802-1813.
4. Li Y, Wang Y, Wu Q, Li L, Shi Y, et al. (2016) Comparison of methods for isolating primary hepatocytes from mini pigs. Xenotransplantation 23: 414-420.
5. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43: 506-520.
6. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Trump BF (1981) Mouse Liver Cell Culture. I. Hepatocyte Isolation In Vitro 17: 913.
7. Jiang QD, Li HP, Liu FJ, Wang XJ, Guo YJ, et al. (2013) Isolation and identification of bovine primary hepatocytes. GMR 12: 5186-5194.

8. Li J, Li LJ, Chao HC, Yang Q, Liu XL, et al. (2005) Isolation and short term cultivation of swine hepatocytes for bioartificial liver support system. Hepatobiliary Pancreat Dis Int 4: 249-253.
9. Sagias FG, Mitry RR, Hughes RD, Lehec SC, Patel AG, et al. (2010) N-acetylcysteine improves the viability of human hepatocytes isolated from severely steatotic donor liver tissue. Cell Transplant 19: 1487-1492.
10. McIntosh MB, Corner SM, Amiot BP, Nyberg SL (2009) Engineering analysis and development of the spheroid reservoir bioartificial liver. Conf Proc IEEE Eng Med Biol Soc 2009: 5985-5988.
11. Sakai Y, Naruse K, Nagashima I, Muto T, Suzuki M (1995) Rapid and Large-Scale Preparation of Porcine Hepatocyte Spheroids and Their Functions in Continuous Suspension Culture. Springer Netherlands.
12. Puviani AC, Ottolenghi C, Tassinari B, Pazzi P, Morsiani E (1998) An update on high-yield hepatocyte isolation methods andon the potential clinical use of isolated liver cells. Comp Biochem Physiol A Mol Integr Physiol 121: 99-109.
13. Malik R, Selden C, Hodgson H (2002) The role of non-parenchymal cells in liver growth. Semin Cell Dev Biol 13: 425-431.
14. Wang Y, Cui CB, Yamauchi M, Miguez P, Roach M, et al. (2011) Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. Hepatology 53: 293-305.
15. Luebke-Wheeler JL, Nedredal G, Yee L, Amiot BP, Nyberg SL (2009) E-cadherin protects primary hepatocyte spheroids from cell death by a caspase-independent mechanism. Cell Transplant 18: 1281-1287.
16. Meier RPH, Navarro-Alvarez N, Morel P, Schuurman H-J, Strom S, et al. (2015) Current status of hepatocyte xenotransplantation. Int J Surg 23: 273-279.
17. Seglen PO (1972) Preparation of rat liver cells. I. Effect of Ca2+ on enzymatic dispersion of isolated, perfused liver. Exp Cell Res 74: 450-454.
18. Bartlett DC, Hodson J, Bhogal RH, Youster J, Newsome PN (2014) Combined use of N -acetylcysteine and Liberase improves the viabilityand metabolic function of human hepatocytes isolated fromhuman liver. Cytotherapy 16: 800-809.
19. Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MT, et al. (1986) Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell Dev Biol 22: 201-211.
20. Edwards AM (1991) Isolated Hepatocytes: Preparation, Properties and Applications. Institucional.
21. Morsiani E, Fogli L, Gorini P, Ricci D, Mazzoni M (1985) Preparation and allotransplantation of isolated hepatocytes in partially hepatectomized rats. Ital J Surg Sci 15: 23.
22. Strain AJ, Mcgowan JA, Bucher NLR (1982) Stimulation of DNA Synthesis in Primary Cultures of Adult Rat Hepatocytes by Rat Platelet-Associated Substance(s). In Vitro 18: 108-116.
23. Skardal A, Smith L, Bharadwaj S, Atala A, Soker S, et al. (2012) Tissue specific synthetic ECM hydrogels for 3-D in vitro maintenance of hepatocyte function. Biomaterials 33: 4565.
24. Pazzi P, Puviani AC, Dalla LM, Guerra G, Ricci D, et al. (1997) Bile salt-induced cytotoxicity and ursodeoxycholate cytoprotection: in-vitro study in perifused rat hepatocytes. Eur J Gastroenterol Hepatol 9: 703.
25. Sellaro TL, Ranade A, Faulk DM, Mccabe GP, Dorko K, et al. (2010) Maintenance of Human Hepatocyte Function In Vitro by Liver-Derived Extracellular Matrix Gels. Tissue Eng Part A 16: 1075.
26. Skardal A, Smith L, Bharadwaj S, Atala A, Soker S, et al. (2012) Tissue specific synthetic ECM hydrogels for 3-D in vitro maintenance of hepatocyte function. Biomaterials 33: 4565.
27. Imai K, Hanaoka K, Mine T (2003) Zonal differences in effects of HGF/SF and EGF on DNA synthesis in hepatocytes at different times post-hepatectomy. Hepatol Res 27: 302-308.
28. Luebkewheeler JL, Nedredal G, Le Y, Amiot BP, Nyberg SL (2009) E-Cadherin Protects Primary Hepatocyte Spheroids from Cell Death by a Caspase-Independent Mechanism. Cell Transplan 18: 1281.
29. Bao J, Fisher JE, Lillegard JB, Wang W, Amiot B, et al. (2013) Serum-free medium and mesenchymal stromal cells enhance functionality and stabilize integrity of rat hepatocyte spheroids. Cell Transplant 22: 299-308.
30. Hickey R, Mao SA, Glorioso J, Elgilani F, Amiot B (2016) Curative ex vivo liver-directed gene therapy in a pig model of hereditary tyrosinemia type 1. Sci Transl Med 8: 349.
31. Gupta S, Vemuru RP, Lee CD, Yerneni PR, Aragona E, et al. (1994) Hepatocytes exhibit superior transgene expression after transplantation into liver and spleen compared with peritoneal cavity or dorsal fat pad: implications for hepatic gene therapy. Hum Gene Ther 5: 959-967.
32. Ito M, Nagata H, Yamamoto T, Yoshihara D, Fox IJ, et al. (2007) Intrasplenic hepatocyte transplantation prolonged the survival in Nagase analbuminemic rats with liver failure induced by common bile duct ligation. Cell Transplant 16: 547-553.
33. Rozga J, Morsiani E, Lepage E, Moscioni AD, Demetriou AA, et al. (1994) Isolated hepatocytes in a bioartificial liver: A single group view and experience. Biotechnol Bioeng 43: 645-653.
34. Lindblad B, Lindstedt S, Steen G (1977) On the enzymic defects in hereditary tyrosinemia. Proc NatL Acad Scd 74: 4647-4645.
35. Endo F, Sun M-S (2002) Tyrosinaemia type I and apoptosis of hepatocytes and renal tubular cells. J Inherit Metab Dis 25: 227-234.
36. Hickey RD, Mao SA, Glorioso J, Elgilani F, Amiot B, et al. (2016) Curative ex vivo liver-directed gene therapy in a pig model of hereditary tyrosinemia type 1. Sci Transl Med 8: 349ra99.
37. Lindstedt S, Holme E, Lock EA, Hjalmarson O, Strandvik B (1992) Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet 340: 813.
38. Hickey RD, Mao SA, Glorioso J, Lillegard JB, Fisher JE, et al. (2014) Fumarylacetoacetate hydrolase deficient pigs are a novel large animal model of metabolic liver disease. Stem Cell Res 13: 144-153.