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***Basic Study***

**Hepatocytes isolation from resected benign tissues: Results of a 5-year experience**

Meng FY *et al.* Hepatocytes isolation from resected tissues

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**Abstract**

***AIM***

To analyze retrospectively a 5-year experience of human hepatocyte isolation from resected liver tissues with benign disease.

***METHODS***

We herein established a method of modified four-step retrograde perfusion to isolate primary human hepatocytes. Samples were collected from the resected livers of patients with intrahepatic duct calculi (*n* = 7) and liver hemangioma (*n* = 17). Only the samples weighed ≥ 15 g were considered suitable for hepatocyte isolation. By using the standard trypan blue exclusion technique, hepatocyte viability and yield were immediately determined after isolation.

***RESULTS***

Twenty-four liver specimens, weighing 15–42 g, were immediately taken from the margin of the removed samples and transferred to the laboratory for hepatocyte isolation. Warm ischemia time was 5–35 min and cold ischemia time was 15–45 min. For the 7 samples of intrahepatic duct calculi, the method resulted in a hepatocyte yield of 3.49 ± 2.31 × 106 hepatocytes/g liver, with 76.4 ± 10.7% viability. In the 17 samples of liver hemangioma group, significantly higher yield of cells (5.4 ± 1.71 × 106 *vs* 3.49 ± 2.31 × 106 cells/g, *P <* 0.05) than in samples of intrahepatic duct calculi were found. However, there seems no clearly difference in cell viability (80.3% ± 9.67% *vs* 76.4% ± 10.7%, *P >* 0.05). In our study, we get a cell yield of 5.31 ± 1.87 × 106 hepatocytes/g liver when the samples weighed > 20 g. But for the tissue weighed ≤ 20 g, a reduction in yields was found (3.08 ± 1.86 × 106 *vs* 5.31 ± 1.87 × 106, *P <* 0.05).

***CONCLUSION***

Benign-diseased livers are valuable sources for large number hepatocyte isolation. Our study represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We evaluated the effect of donor liver characteristics on cell isolation, and we found that samples of liver hemangioma can get better results in terms of cell yield than intrahepatic duct calculi. Furthermore, the size of the tissues can affect the outcome of hepatocyte isolation.

**Key words:** Human hepatocyte; Primary hepatocyte; Cell isolation; Benign liver disease; Hepatocyte isolation

**Core tip:** We retrospectively analyze a 5-year experience of human hepatocyte isolation from surgically resected normal tissue and established an efficient technique for the special kind of liver samples for large-scale human hepatocyte isolation. Our study represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We evaluated the effect of donor liver characteristics on cell isolation, and we found that samples of liver hemangioma can get better results in terms of cell yield than intrahepatic duct calculi. Furthermore, the size of the tissues can affect the outcome of hepatocyte isolation.

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**INTRODUCTION**

Demands for primary human hepatocytes for basic research and therapeutic applications are continuously increasing[1-3]. Large quantities of primary human hepatocytes can be isolated mainly from two sources: discarded liver transplants, and liver specimens obtained during partial hepatectomy[4,5]. Compared to organs from discarded liver transplants, the latter are easily accessible and more frequently available. Furthermore, resected liver donations are usually planned and often occur during normal working hours. Nevertheless, surgically resected tissue is of varying quality, which can affect the yield and viability of isolated hepatocytes[6]. It is important to standardize the use of the surgical specimens to maximize the availability of high-quality hepatocytes.

Some authors have previously suggested protocols for human hepatocyte isolation from surgically resected liver tissues[5,7,8]. However, the majority of these groups have used tissue from malignant tumors[9]. Often totally healthy tissues are needed for clinical application of hepatocyte transplantation, artificial liver, and hepatocyte immortalization[10,11]. There are still no systematic studies investigated in resected specimens from patients with benign liver disease for large-scale hepatocyte isolation. Therefore, available data about the effect of the donor liver on isolated hepatocyte yield are scarce. To address these issues, we retrospectively analyze a 5-year experience of human hepatocyte isolation from surgically resected normal tissue and evaluated the effect of donor liver characteristics on hepatocyte isolation outcome. Our study herein represents the largest number of primary human hepatocytes isolated from resected specimens from patients with benign liver disease. We established an efficient technique for the special kind of liver samples for large-scale human hepatocyte isolation. The aim of our study was to rescue resected healthy liver tissue that would be otherwise discarded, for hepatocyte isolation. We also compared the two different kind of liver samples resulted in different cell yield and viability.

**MATERIALS AND METHODS**

***Surgery and tissue collection***

We chose patients with liver benign disease, intrahepatic duct calculi and liver hemangioma, performed in our hospital. Following institutional and ethical guidelines and after obtaining tissue donors’ consent in our hospital, samples weighed ≥ 15 g were collected from 24 patients undergoing partial hepatectomy, 7 liver intrahepatic duct calculi and 17 liver hemangioma (Table 1). To avoid unnecessary damage to the hepatocytes, continuous clamping was applied in all 24 cases, with a short intraoperative warm ischemia time (5–35 min). After the liver tissues (15–42 g) were resected from the abdominal cavity, they were immediately placed into ice-cold Ringer’s lactate solution under sterile conditions. The tissues were directly transferred to the laboratory for hepatocyte isolation, with a limited cold ischemic time (15-45 min).

***Perfusion apparatus setup***

To ensure flow of appropriate buffers, a peristaltic pump with adjustable speed was used in our perfusion system. This system was formed by mounting a peristaltic pump with two silicone tubings immersed in a variable-sized water tank to accommodate the liver tissues. The water bath temperature was adjusted and maintained at 37 °C for liver undergoing perfusion and digestion. The pump speed was set at constant flow.

***Reagents and solutions preparation***

The method used several reagents that were prepared in advance (Table 2). The reagents mixed thoroughly, filter solutions prepared using 0.22-µm filters, pH value adjusted to 7.4, and stored at 4 °C.

***Hepatocyte isolation***

We adopted a rigorous and stringent isolation protocol for all liver tissues. Precise time between tissue resection and isolation commencement was recorded. Liver wedges were usually obtained from segments II/III and were carefully cut and weighed. Primary human hepatocytes were isolated under strict sterile conditions using a modified four-step retrograde perfusion technique (Figure 1). To eliminate interpersonal variability, all of the subsequent isolation procedures were carried out by single individual (Meng FY). The liver samples were cannulated into the main blood vessel on the cut surface. The liver tissue was flushed with ice-cold PBE using blood vessels on the cut surface. This step was important to remove excess blood and help to determine the vessels that would offer optimal perfusion subsequently. The chosen vessel was cannulated with a suitable pipette tip, and flushed with PBE at 37 °C. In some cases, there might be more than one cut surface, or there might be a cut or tear on the outer capsule of the liver tissue (Glisson’s capsule). These must be sewed up in advance to ensure optimal perfusion of the tissue.

After flushing with PB to clear PBE, the tissue was then continuously perfused with a prewarmed digestion buffer solution (PBD and PBC). After sufficient digestion, the liver Glisson’s capsule was mechanically disrupted by using an operating knife blade. The isolated hepatocytes were released into the medium by gently shaking, leaving behind the connective tissue and any undigested material. The resultant hepatocyte suspension was divided equally into sterile centrifuge bottles. The suspension was filtered through a 500- um nylon mesh and centrifuged at 50 × *g* for 2 min at 4 °C. We regularly used a 10-min cell incubation step by using buffer solution containing DNase I (WBD). Cell clumps were broken up and damaged cells were digested. The suspension was then filtered (75 s), and the resultant cells were harvested by low-speed centrifugation at 50 x *g* for 75 s. This was followed by washes in cold buffer solution (WB), filtration (60 s) and another centrifugation step (50 × *g*, 75 s, 4 °C). Finally, the resultant hepatocyte clumps were resuspended in cold William’s Medium E (Sigma). Hepatocyte yield and viability were immediately determined using the standard trypan blue exclusion technique after isolation/purification.

***Hepatocyte culture***

Culture medium was William’s E Medium supplemented with 100 μg/mL streptomycin, 100 mU/mL penicillin and 10% fetal bovine serum (FBS). Freshly isolated hepatocytes were seeded in culture flasks at a concentration of 4 × 105 to 5 × 105/mL. The culture medium was changed every 24 h. The morphology of the cultured cells was assessed throughout the entire culture period using light microscopy. Phase-contrast microscopy pictures were taken with a Nikon Diaphot inverted microscope.

**RESULTS**

The type of liver tissues used and the hepatocytes isolated are shown in Table 1. The liver tissue donors had intrahepatic duct calculi (*n* = 7) and liver hemangioma (*n* = 17). To improve cell availability, we investigate the influence of warm and cold ischemia time and liver donor characteristics on the outcome of freshly isolated hepatocytes from surgically resected liver tissue. All patients (15 male and 9 female) were negative for hepatitis C virus (HCV), hepatitis B virus surface antigen (HBsAg), and human immunodeficiency virus (HIV). All the patients had normal liver function tests prior to surgery. Mean donor age was 50.7 years (range: 23–79 years). Blood group was O+ in 8 cases, A+ in 9, AB+ in 1, and B+ in 6 (Table 1). Data from our work revealed that patient sex, age and blood group had no correlation with cell yield and viability.

Liver wedges were prepared as shown in Figure 2. Representative images of isolated primary human hepatocytes, in culture for the first week, are shown in Figure 3. Similar morphological changes in the cultured cells were observed during the first week. They maintained normal morphology for at least 1 wk during culture in William’s E Medium.

We processed and isolated human hepatocytes from 24 liver wedges. The warm ischemia time (WIT), *i.e*., the interval between clamping and bathing in ice-cold solution, averaged 17.5 ± 8.8 min (range: 5–35 min) (Table 1). The cold ischemia time, *i.e*., the interval between liver resection and perfusion, averaged 19.3 ± 3.3 min (range: 15–45 min). For the 7 samples of intrahepatic duct calculi, the method resulted in a hepatocyte yield of 3.49 ± 2.31 × 106 hepatocytes/g liver, with 76.4% ± 10.7% viability. However, for the 17 samples of liver hemangioma, we got better results with a hepatocyte yield ( 5.4 ± 1.71 × 106 cells/g *vs* 3.49 ± 2.31 × 106 cells/g, *P <* 0.05) than in samples of intrahepatic duct calculi were found (Figure 4A). However, there seems no clearly difference in cell viability (80.3% ± 9.67% *vs* 76.4% ± 10.7%, *P >* 0.05) (Figure 4B). In our study, we get a cell yield of 5.31± 1.87 × 106 hepatocytes/g liver when the samples weighed > 20 g. But for the tissue weighed ≤ 20 g, a reduction in yields was found (3.08 ± 1.86 × 106 *vs* 5.31 ± 1.87 × 106, *P <* 0.05) (Figure 4C). And it seems no difference in cell viability (80.0 ± 9.85% *vs* 76.0 ± 10.5%, *P >* 0.05) (Figure 4D)

All of the cultured primary hepatocytes demonstrated albumin synthesis in the first week (Table 3). Serum albumin concentrations were determined using immunonephelometry (Array; Beckman Instruments, Galway, Ireland). The isolated hepatocytes showed significantly increased albumin synthesis after 2 d culture. When human hepatocytes were cultured in William’s E Medium supplemented with 10% FBS, the cells maintained their polygonal shape until day 5 (Figure 3).

**DISCUSSION**

Hepatocyte isolation is a time-consuming and costly procedure[12,13]. However, surgical specimens are of varying quality, which can affect the cell yield and viability. It is important to identify surgically resected tissues with the best expectations concerning cell quality and yield[14-16]. The aim of the present study was to analyze retrospectively the influence of the donor liver characteristics on the outcome of primary hepatocyte isolation obtained from surgically resected liver tissue. Once such a standardized method has been established, isolated hepatocytes would be easily accessible and more frequently available. Hepatocyte isolation started in the mid-1960s[17]. After that, a lot of innovative techniques were introduced to improve the results[8,18,19]. However, most of the innovative techniques have been applied exclusively to tissue obtained from resected liver tumors or from whole organ donors[20]. We established a modified four-step collagenase retrograde perfusion technique for isolation of hepatocytes from non-diseased liver tissue removed at surgical resection. The modified method, compared to the traditional method, can improve results, allowing isolation of a large number of hepatocytes of high quality. After hepatocyte isolation, a 10-min incubation step using DNase I (WBD) was used. Cell clumps break up and damaged cells are digested. Our technique resulted in a hepatocyte yield of 4.85 ± 2.05 × 106 cells/g liver. The viability of the isolated hepatocytes, using the trypan blue exclusion technique, was 79.17% ± 9.90%.

Intrahepatic duct calculi and liver hemangioma are two common diseases among the local population. Tissues from patients undergoing partial hepatectomy are the most frequently available sources for hepatocyte isolation. However, not all of these tissues can be used to isolate large numbers of hepatocytes. Hepatic fibrosis often occurs in patients with intrahepatic duct calculi, who generally do not yield successful cell isolation. Different with other experiences reported in the literature[21], our results showed in the 17 samples of liver hemangioma group, significantly higher yield of cells (5.4 ± 1.71 × 106) than in samples of intrahepatic duct calculi (3.49 ± 2.31 × 106 cells/g, *P <* 0.05). However, there seems no clearly difference in cell viability (80.3% ± 9.67% *vs* 76.4% ± 10.7%, *P >* 0.05). Cytotoxic bile acids accumulate in the hepatocytes during cholestasis, which is thought to induce hepatocyte necrosis and contribute to development of liver cirrhosis[22,23]. This could explain the lower cell yield observed in hepatocytes isolated from cholestatic livers[24-26]. In contrast, Iqbal *et al*[27] observed no significant difference in cell yield and viability in hepatocytes isolated from resected cirrhotic compared to noncirrhotic livers. Further investigation should be made to evaluate the usage of resected cirrhotic livers for cell isolation.

Compared to resected livers from patients with intrahepatic duct calculi, we more frequently obtained a reliable source of normal liver tissue from patients with hemangioma. Furthermore, we found that left lateral sector segments were usually suitable to obtain normal resected tissues, with proportionate volumes and then the lobular blood vessels can be easily exposed for catheterization. Of the total 24 samples, 19 were got from left hemihepatectomy. The fragments of hepatic tissue were cut from the periphery of the discarded material, surrounded by hepatic capsule. 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. We consider that the first perfusion step to drain off blood in the vessels is critical, because it can significantly affect the outcome of the subsequent collagenase digestion.

Among the general features of the patient, blood group, age and sex, did not have any influence on the yield or viability of the isolated human hepatocytes. In contrast, several investigations have described a decrease in hepatocyte viability or yield with an increase in patient age[8,28]. This may have resulted from the distribution of diseases among the different age groups, which can significantly affect the outcome of hepatocyte isolation[8]. Patients aged > 50 years were mainly malignant diseases. In our study, all of the specimens were obtained from patients with benign liver disease. Without the interfere factors, we observed that patient age had no correlation with cell yield and viability.

Warm ischemia time can affect the outcome of hepatocyte isolation. It is reported that porta hepatis clamping during liver resection results in a low yield of isolated hepatocytes[26]. There is a negative correlation between warm ischemia time (< 30 min) and low cell yield[26]. It is reported that intermittent clamping is more damaging to the liver than continuous clamping[29]. As for cold ischemia time, it is reported that ≤ 24 h has no effect on hepatocyte yield and viability[28,30,31]. As shown in our study, when resected liver tissue is used for hepatocyte isolation, the warm and cold ischemia time are always short. So, warm and cold ischemia time seem to have no obvious effect on the outcome of hepatocyte isolation from surgically resected tissue.

The size of the tissue can affect the outcome of hepatocyte isolation. Alexandre *et al*[26] concluded that the percentage of digested liver decreased when tissue weights were > 100 g. In our study, we found a reduction in yields when the tissue weighed ≤ 20 g (Table 2). One reason is that small tissue samples always have several cut surfaces without an integrated hepatic capsule. Another reason is that it is difficult to find a visible vessel orifice on the cut surface to perfuse collagenase solutions. In contrast to the large tissue samples, small samples always have a longer isolation process with insufficient digestion. It suggests that the four-step perfusion technique for tissues ≤ 20 g should be modified. One possible method to improve the isolation outcome of small tissue samples is to glue the section surfaces in order to avoid leakage of the perfusate. However, some studies have reported that the use of glue tends to decrease the yield of viable cells[26]. Another possible method to increase the hepatocyte yield is to separate the viable cells using a Percoll centrifugation technique. We need to consider whether Percoll centrifugation is acceptable for the large quantity of hepatocytes required for clinical application. Due to the small numbers of tissues in our study, further investigations are needed to evaluate the optimal procedure for small resected specimens.

In conclusion, benign-diseased livers appear to be a valuable source of a large number of isolated human hepatocytes. We consider that patient age has no correlation with cell yield and viability. Mild cirrhotic livers should not be arbitrarily excluded from cell isolation. We recommend, for optimal isolation, to use liver specimens weighing > 20 g, avoid the use of liver specimens with severe fibrosis.

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**COMMENTS**

***Background***

There are still no systematic studies investigated in resected specimens from patients with benign liver disease for large-scale hepatocyte isolation. Therefore, available data about the effect of the donor liver on isolated hepatocyte yield are scarce.

***Research frontiers***

The authors established an efficient technique for the special kind of liver samples for large-scale human hepatocyte isolation. They retrospectively analyze a 5-year experience of human hepatocyte isolation from surgically resected normal tissue and evaluated the effect of donor liver characteristics on hepatocyte isolation outcome.

***Innovations and breakthroughs***

The authors consider that patient age has no correlation with cell yield and viability. Mild cirrhotic livers should not be arbitrarily excluded from cell isolation. They recommend, for optimal isolation, to use liver specimens weighing > 20 g, avoid the use of liver specimens with severe fibrosis.

***Applications***

Benign-diseased livers appear to be a valuable source of a large number of isolated human hepatocytes.

***Terminology***

WIT (warm ischemia time), the interval between clamping and bathing in ice-cold solution. CIT (warm ischemia time), the interval between storing in ice-cold solution and starting isolation.

***Peer-review***

The manuscript is well written. The study conducted for 5 years is well described and The results are clear and explain in a good manner The target of The authors research.

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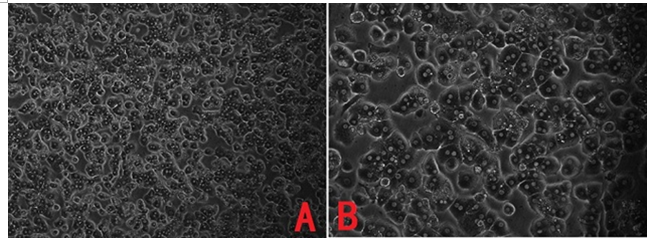
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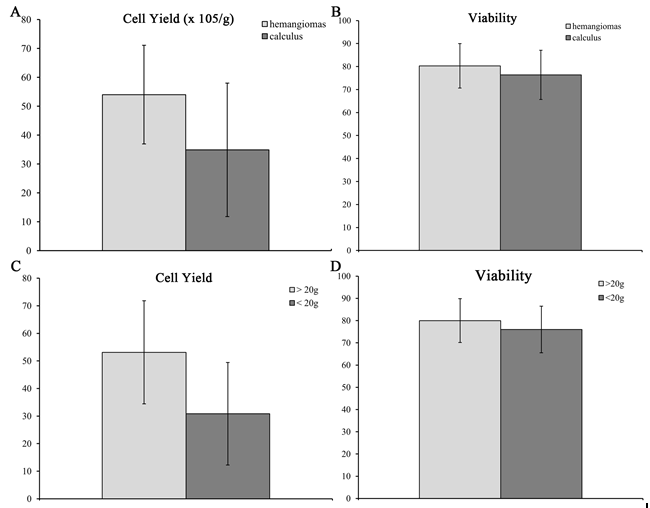
**Figure 1 Flow diagram of the preparation of isolated human hepatocytes with a modified four-step retrograde perfusion technique.** Buffers**:** PBE: Perfusion buffer with EDTA; PB: Perfusion buffer; PBD: Perfusion buffer with dispase; PBC: Perfusion buffer with collagenase; WB: Washing buffer; WBD: Washing buffer with DNase.



**Figure 2 Preparation of liver wedges.** Only patients who had undergone left hemihepatectomies were deemed suitable to obtain normal resected liver tissue from (A). Cut off end of a suitable pipet tip to obtain optimal size to match vessel opening and cannulate the chosen vessel with it (B, C). Primary human hepatocytes must be isolated under stringent and rigorous sterile conditions (D).



**Figure 3 Phase-contrast photographs of primary human hepatocytes 24 h after isolation.** Magnification× 100 (A) and × 200 (B).



**Figure 4 Samples of liver hemangioma can get better results in terms of cell yield than intrahepatic duct calculi.** And the size of the tissues can affect the outcome of hepatocyte isolation.

**Table 1 Patient profile and isolated human hepatocyte viability, total cell yield and hepatocyte yield**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **No.** | **Sex** | **Age (yr)** | **Blood group** | **Disease** | **Tissue weight (g)** | **WIT (min)** | **Hepatocyte (× 105/g)** | **Viability (%)** |
| 1 | Male | 48 | O+ | Hemangiomas | 15 | 30 | 28.00 | 62 |
| 2 | Male | 52 | A+ | Hemangiomas | 18 | 25 | 20.56 | 73 |
| 3 | Female | 23 | A+ | Calculus | 21 | 25 | 12.86 | 69 |
| 4 | Male | 55 | O+ | Hemangiomas | 26 | 30 | 18.08 | 65 |
| 5 | Female | 45 | A+ | Hemangiomas | 27 | 25 | 51.85 | 76 |
| 6 | Female | 36 | B+ | Hemangiomas | 32 | 15 | 65.63 | 85 |
| 7 | Male | 47 | AB+ | Calculus | 38 | 10 | 71.05 | 92 |
| 8 | Male | 42 | A+ | Hemangiomas | 42 | 10 | 54.76 | 86 |
| 9 | Male | 52 | A+ | Hemangiomas | 20 | 5 | 63.30 | 87 |
| 10 | Male | 63 | O+ | Hemangiomas | 33 | 25 | 72.11 | 93 |
| 11 | Female | 46 | O+ | Hemangiomas | 27 | 20 | 43.70 | 68 |
| 12 | Male | 57 | A+ | Hemangiomas | 30 | 35 | 55.21 | 75 |
| 13 | Male | 44 | B+ | Calculus | 25 | 15 | 63.81 | 84 |
| 14 | Male | 61 | A+ | Calculus | 17 | 5 | 25.20 | 86 |
| 15 | Female | 57 | A+ | Emangiomas | 32 | 7 | 62.50 | 89 |
| 16 | Male | 27 | A+ | Emangiomas | 27 | 15 | 52.65 | 76 |
| 17 | Female | 55 | B+ | Calculus | 23 | 7 | 32.07 | 66 |
| 18 | Male | 61 | O+ | Emangiomas | 31 | 25 | 68.80 | 90 |
| 19 | Male | 37 | B+ | Emangiomas | 37 | 21 | 72.22 | 92 |
| 20 | Male | 65 | B+ | Calculus | 20 | 15 | 17.07 | 72 |
| 21 | Male | 79 | O+ | Calculus | 26 | 20 | 22.51 | 66 |
| 22 | Male | 47 | O+ | Emangiomas | 35 | 5 | 67.72 | 89 |
| 23 | Male | 51 | B+ | Emangiomas | 24 | 12 | 59.27 | 77 |
| 24 | Male | 67 | O+ | Emangiomas | 30 | 17 | 62.25 | 82 |

**Table 2 Reagents and solutions preparation (prepared for 80 g liver sample)**

|  |  |  |
| --- | --- | --- |
| **Resolution** | **Ingredient** | **Concentration (g/L)** |
| PB (Perfusion buffer) | Double-distilled water (3000 mL) |  |
| NaCl (27 g) | 9 |
| KCl (1.26 g) | 0.42 |
| NaHCO3 (6.3 g) | 2.1 |
| Glucose (2.7 g) | 0.9 |
| Hepes (14.34 g) | 4.78 |
| PBE (Perfusion buffer with EDTA) | PB (1000mL) |  |
| EDTA (0.37 g) | 0.37 |
| PBD (Perfusion buffer with Dispase) | PB (500 mL) |  |
| Dispase II *(*Sigma) (4.2 g) | 8.4 |
| PBC (Perfusion buffer with Collagenase) | PB (500 mL) |  |
| Collagenase IV*(*Sigma) (0.25 g) | 0.5 |
| CaCl2·2H2O (0.275 g) | 0.55 |
| WB (Washing buffer) | Double-distilled water (2500 mL) |  |
| NaCl (17.5 g) | 7 |
| KCl (1.15 g) | 0.46 |
| CaCl2·2H2O (0.325 g) | 0.13 |
| Hepes (5.95 g) | 2.38 |
| Bovine serum albumin (2.5 g) | 1.0 |
| WBD (Washing buffer with DNase) | WB (Washing buffer) (1500 mL) |  |
| MgCl2·6H2O (0.15 g) | 0.1 |
| MgSO4·7H2O (0.15 g) | 0.1 |
| DNase I *(*Sigma) (0.15 g) | 0.1 |

**Table 3 The concentrations of albumin in the cultured hepatocytes suspension**

|  |  |
| --- | --- |
| **Time** | **Albumin (g/L)** |
| 24 h | 0.79 ± 0.31 |
| 7 d | 1.36 ± 0.42 |
| 10 d | 1.09 ± 0.21 |

The concentration of albumin in the William’s E culture medium is about 0.5 g/L.