Video Article

Isolation of Human Hepatocytes by a Two-step Collagenase Perfusion Procedure

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Abstract

The liver, an organ with an exceptional regeneration capacity, carries out a wide range of functions, such as detoxification, metabolism and homeostasis. As such, hepatocytes are an important model for a large variety of research questions. In particular, the use of human hepatocytes is especially important in the fields of pharmacokinetics, toxicology, liver regeneration and translational research. Thus, this method presents a modified version of a two-step collagenase perfusion procedure to isolate hepatocytes as described by Seglen [1].

Previously, hepatocytes have been isolated by mechanical methods. However, enzymatic methods have been shown to be superior as hepatocytes retain their structural integrity and function after isolation. This method presented here adapts the method designed previously for rat livers to human liver pieces and results in a large yield of hepatocytes with a viability of 77±10%. The main difference in this procedure is the process of cannulization of the blood vessels. Further, the method described here can also be applied to livers from other species with comparable liver or blood vessel sizes.

Video Link

The video component of this article can be found at http://www.jove.com/video/50615/

Introduction

A liver cell suspension can be prepared from the liver by mechanical or enzymatic methods. Mechanical methods used to prepare whole liver cells include forcing the liver through cheesecloth [2], shaking a liver piece with glass beads in a Kahn shaker [3], using glass homogenizers with loose pestles [4,5] *etc.* Over the years, mechanical methods have fallen out of favor due to the damage to cell membranes and the loss of function of the isolated hepatocytes [6,7]. Consequently, the use of an enzymatic method is currently the main method for isolation of hepatocytes.

Isolation of hepatocytes using an enzymatic method was greatly improved when Berry and Friend [8] perfused collagenase and hyaluronidase through the liver via the portal vein in rats. This perfusion process utilized the vasculature to allow the enzymes to come into close contact with the majority of the cells, leading to a 6-fold increase in yield of hepatocytes [8]. Further, this method yielded cells that retained their structural integrity, with virtually no transformation of endoplasmic reticulum into isolated vesicles and no mitochondrial damage [8].

This method was modified by Seglen [1], who pioneered a two-step perfusion procedure for liver cell isolation. In this procedure, the rat liver is perfused with a Ca^{2+} free buffer followed by perfusion with a collagenase buffer containing Ca^{2+} [1]. The removal of Ca^{2+} in the first step helps to disrupt desmosomes, while the addition of Ca^{2+} in the second step is required for optimum collagenase activity [1,9].

Given that the published work described above has been performed in rats, this article aims to demonstrate a modified procedure that can be used for isolation of hepatocytes with high viability from human livers. The use of human hepatocytes remains important for translational research and for validating experiments using animal models. The human liver pieces used in this study were acquired with consent for governance through the Human Tissue and Cell Research Foundation, a state-controlled non-profit foundation [10]. After a pathologist removed what was required for diagnosis, liver pieces were collected from the remaining tissue. The tissue sectioned off by the pathologist was morphologically healthy tissue obtained from resection margins after liver resection.

Protocol

1. Preparation of Perfusion and Isolation Solutions

1. Prepare the solutions required for the perfusion of the liver piece and the isolation of hepatocytes according to **Table 1**. Solutions can be stored at 4 °C until use.
2. Sterile filter all solutions using a 0.22 µm filter.
3. All solutions that come into contact with the liver should be sterile.

2. Preparation of Perfusion Equipment and Solutions

1. The equipment for the perfusion of the liver piece should be set up as shown in **Figure 1**.
2. The water bath should be set at an appropriate temperature, which is different in each particular experimental set-up, such that the solutions are at the temperature of 37 °C when they reach the liver piece. In this case, the water bath is set at 41 °C to warm the Solutions 1, 2 and 3 and the jacketed glass condenser. Solution 4 should be warmed up to 37 °C in a separate water bath for use to reduce the loss of collagenase activity.
3. Shortly before liver perfusion, turn on the regulator of the gas tank containing 95% O_2/5% CO_2 to gas the oxygenation apparatus (**Figure 1E**).

3. Perfusion of the Liver

1. A liver piece with as much intact Glisson's capsule as possible and ideally with only 1 cut surface should be obtained from a pathologist for perfusion.
2. Place this liver piece on the Büchner funnel that contains a perforated filter disc (**Figure 1B**).
3. The perfusion system should be primed with Solution 1.
4. With a low flow rate, curved irrigation cannulae with olive tips should be inserted into the larger blood vessels on the cut surface of the liver piece. As blood flushes out from the liver, the tissue becomes lighter in areas with good perfusion. The number of cannulae used for various sizes of livers is shown in **Figure 2A**. The gauge size chosen should result in a snug fit that will hold the cannulae in place. The smaller blood vessels should be left open for the perfusion buffer to drain out of the liver piece.
5. Increase the flow rate on the peristaltic pump to between 110-460 ml/min depending on the size of the liver (**Figure 2B**). This results in an average flow rate of 44±16 ml/min per cannula (**Figure 2C**). The speed chosen depends on the liver piece and should result in a slight plumping up of the liver piece. In some cases, it may be necessary to clamp shut some of the open vessels with micro vascular clamps to achieve the slight plumping mentioned above. A good perfusion can be observed when the liver piece is a lighter color throughout.
6. Keep the liver piece moist during perfusion by covering it with a piece of gauze soaked in saline.
7. Perfuse with 1 L of Solution 1 to flush out any remaining blood in the liver piece.
8. Change the perfusion fluid to Solution 2 and perfuse for 10 min.
9. Switch the perfusion fluid to Solution 3 and perfuse with 0.5 L.
10. Change the perfusion fluid to Solution 4, which contains 0.1-0.15% of collagenase (**Table 2**).
11. For this step, perfusion should be carried out in a recirculating manner for 9-12 min or until the liver is sufficiently digested; the liver tissue should appear to break apart slightly under the Glisson's capsule and feel softened when probed with the blunt side of a scalpel.

4. Isolation of Hepatocytes

1. Turn off the peristaltic pump and remove cannulae from the liver piece.
2. Place the liver piece in a crystallizing dish containing 100-200 ml of Solution 5.
3. Remove the Glisson's capsule carefully and gently shake out the cells. If there are regions that are not well perfused, a scalpel can be used to cut through these regions to release cells contained within. Add more Solution 5 as needed during the process.
4. Add more Solution 5 until a final volume of 500 ml is reached.
5. Filter cell suspension twice; first through a 210 µm nylon mesh followed by a 70 µm nylon mesh. Next, pour the cell suspension into 200 ml centrifuge tubes.
6. Centrifuge the cell suspension at 72 g for 5 min at 4 °C. Aspirate supernatant and gently resuspend cell pellet gently in 200 ml of Solution 5.
7. Repeat the washing step number 4.6 three times. On the final centrifuge step, resuspend cells in cold storage solution (see list of materials). Cells should be approximately 2-5 million hepatocytes per milliliter for assessment of yield and viability using a hemocytometer-based trypan blue exclusion assay.
8. To carry out a trypan blue exclusion assay, add 0.1 ml of appropriately diluted cells (≈2-5 million/ml) to a microfuge tube containing 0.5 ml of trypan blue solution (0.4% trypan blue dissolved in phosphate-buffered saline (PBS)) and 0.4 ml of PBS. After mixing the cell suspension thoroughly, load a hemocytometer with the suspension and examine under a microscope at 100X magnification. Under microscopy, the dead cells will be stained blue while the live cells appear unstained. Count the number of live and total cells in each of the 1 mm^2 grids marked on the hemocytometer. Viability (%), yield of live cells (million hepatocytes per ml cold storage solution (CSS) or million hepatocytes per g liver) can be calculated using the formulae below.

$$\text{Viability (\%)} = \frac{\text{Average number of live cells in 1 mm}^2}{\text{Average total number of cells in 1 mm}^2} \times 100\%$$

$$\text{Yield (million hepatocytes / ml CSS)} = \text{Average number of live cells in 1 mm}^2 \times \text{Trypan blue dilution factor} \times \text{haemocytometer factor}$$

$$\text{Yield (million hepatocytes / g liver)} = \frac{\text{Yield (million hepatocytes / ml CSS)} \times \text{Volume of resuspended cells in Step 4.7 (ml)}}{\text{Weight of perfused liver (g)}}$$

Note: In this case, the trypan blue dilution factor is 10 and the hemocytometer factor is 10,000.

Representative Results

Perfusion Setup

The equipment required for liver perfusion should be set up according to **Figure 1**.

Viability and Yield of Isolated Human Hepatocytes

The average viability of isolated human hepatocytes was 77±10% and the average yield of hepatocytes was 13±11 million hepatocytes/g liver, with values expressed as means ± standard deviation. The number of hepatocyte isolations carried out to obtain these averages was 648 isolations carried out from January 1999 to December 2012.

Suitable Perfusion Parameters

In order to carry out a successful flushing and perfusion of the liver, the number of cannulae used should vary according to the weight of the liver (**Figure 2A**). In general, 4-8 cannulae should be used for livers ranging from below 20 g to over 80 g. A suitable rate of perfusion, which is also dependent on liver weight, should be chosen for a successful perfusion of the liver (**Figures 2B** and **C**). It has been found that an average perfusion speed of 44 ml/min cannula^{-1} is ideal across a range of different liver weights and therefore the perfusion speeds should be adjusted appropriately if more cannulae are used. If perfusion is successful, the liver should be pale in color and slightly plumped up.

Purity of Hepatocytes

By means of immunofluorescence, it was found that isolated hepatocytes, which stain positively for albumin, had a purity of 94±1% (N = 4 with 5 replicates each) (**Figures 3A** and **B**). **Figure 3C** is a representative phase contrast image showing the morphological characteristics of hepatocytes such as large cell size and polygonal shaped-cells.

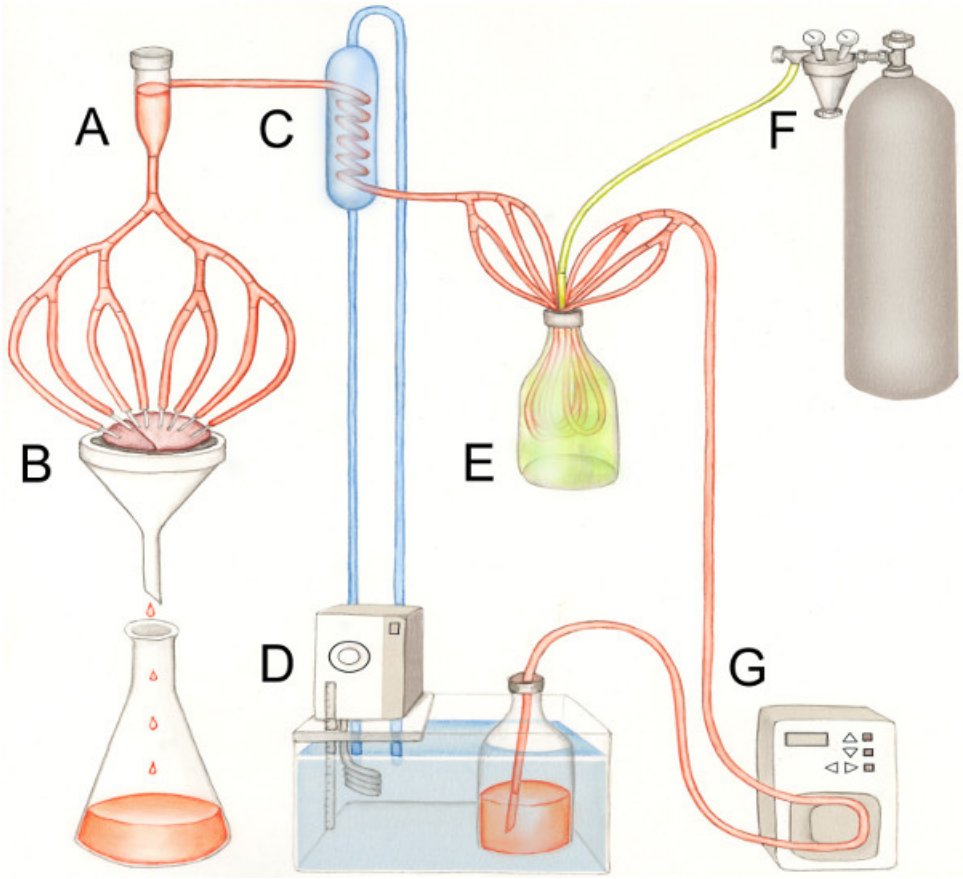

Figure 1. Perfusion setup. (A) bubble trap, **(B)** liver piece with curved irrigation cannulae with olive tips inserted in blood vessels on a Büchner funnel, **(C)** glass jacketed condenser, **(D)** water bath, **(E)** oxygenation apparatus, **(F)** 95% O_2/5% CO_2 gas tank and **(G)** peristaltic pump.

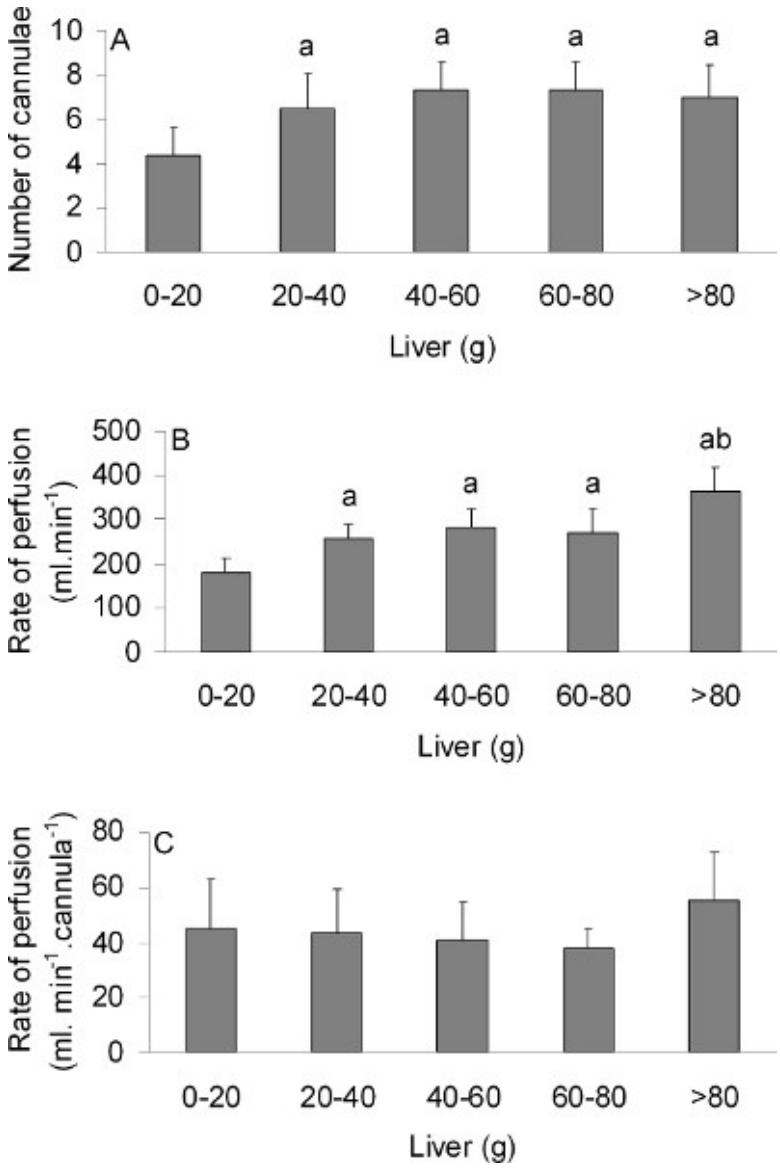

Figure 2. (A) The number of cannulae used, **(B)** perfusion rate (ml/min), or **(C)** perfusion rate (ml/min·cannula) for various sizes of liver (g). Values represent means ± standard deviation with N = 25, 41, 18, 14 and 9 for 0-20 g, 20-40 g, 40-60 g, 60-80 g and >80 g liver respectively. [a]Significantly different from the 0-20 g condition, P<0.05. [ab]Significantly different from the 20-40 g, 40-60 g and 60-80 g condition, P<0.05.

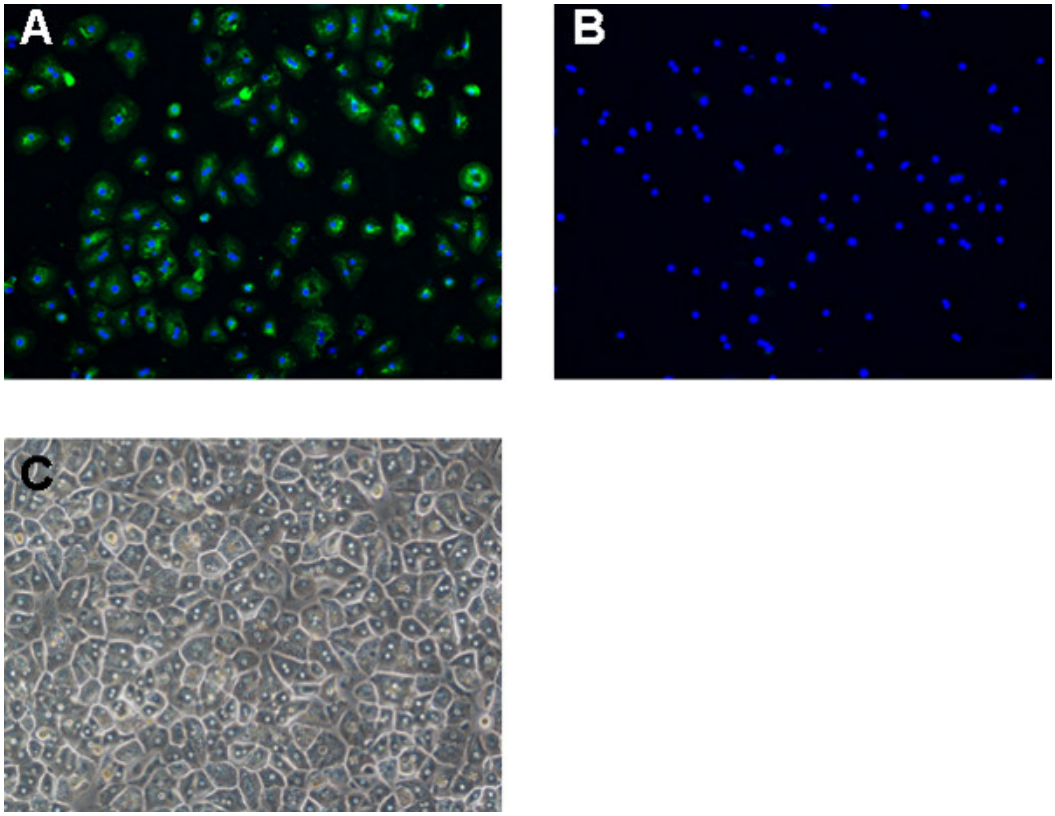

Figure 3. Immunofluorescent images of isolated cells positive for **(A)** albumin (stained in green) and the corresponding **(B)** negative control (200X magnification). Nuclei are stained in blue using DAPI. **(C)** Phase contrast image of isolated cells (100X magnification).

Solution	Constituent	Final Concentration
Solution 1	Sodium chloride	154 mM
	HEPES	20 mM
	Potassium chloride	5.6 mM
	Glucose	5 mM
	Sodium hydrogen carbonate	25 mM
Solution 2	Sodium chloride	152.5 mM
	HEPES	19.8 mM
	Potassium chloride	5.5 mM
	Glucose	5.0 mM
	Sodium hydrogen carbonate	24.8 mM
	EGTA	0.1 mM
To prepare Solution 2, add 10 ml of 100 mM EGTA to 990 ml of Solution 1.		
Solution 3	Sodium chloride	152.5 mM
	HEPES	19.8 mM
	Potassium chloride	5.5 mM
	Glucose	5.0 mM
	Sodium hydrogen carbonate	24.8 mM
	Calcium chloride dihydrate	0.5 µM
To prepare Solution 3, add 10 ml of 0.5 M calcium chloride dihydrate to 990 ml of Solution 1.		
Solution 4	Sodium chloride	152.5 mM
	HEPES	19.8 mM

	Potassium chloride	5.5 mM
	Glucose	5.0 mM
	Sodium hydrogen carbonate	24.8 mM
	Calcium chloride dihydrate	0.5 µM
	Collagenase	See **Table 2**
To prepare Solution 4, add appropriate amount of collagenase to Solution 3.		
Solution 5	Sodium chloride	120 mM
	HEPES	10 mM
	Calcium chloride dihydrate	0.9 mM
	Potassium chloride	6.2 mM
	Albumin	0.1% w/v

Table 1. Perfusion and isolation solutions.

Size of liver piece (g)	Collagenase concentration (%)	Collagenase activity (U/ml)
<25	0.10	250
25 - 40	0.11	300
41 - 80	0.13	350
>80	0.15	400

Table 2. Collagenase concentrations (%) and activities (U/ml) to be used for various sizes of liver pieces (g).

Discussion

This protocol results in the isolation of human hepatocytes with high viability and purity. In order to achieve these results, it is important to start with a suitable piece of liver. The piece of liver should have intact Glisson's capsule on all surfaces except for 1 cut surface. Another important factor is the particular batch of collagenase used, as different batches can result in marked differences in viabilities of hepatocytes after digestion [11]. Therefore, different batches of collagenase should be tested and the batch that produces hepatocytes with the best viability should be obtained in large quantities. Finally, a suitable digestion time has to be chosen based on the yield and viability of the cells obtained. For example, a high viability with low yield could indicate an insufficient digestion time, and a high yield with low viability could indicate that the digestion time is too long.

This method can be adapted to isolate non-parenchymal cells and hepatocytes from the same liver piece. One way of doing this is to use the supernatant from the first centrifugation step (step 4.6) directly for non-parenchymal cell isolation [12]. A second way is to remove the required aliquot of cell suspension after filtration through the nylon mesh for hepatocyte isolation (step 4.5) and subject the remaining cell suspension to an additional pronase digestion step before non-parenchymal cell isolation in order to increase the yield of non-parenchymal cells [13]. For a higher yield of non-parenchymal cells at the expense of the hepatocytes, this method can be modified by substituting collagenase alone (step 3.10) for pronase and collagenase and using the resultant cell suspension for non-parenchymal cell isolation [14].

In addition to isolating human hepatocytes, this method presented can also be adapted to isolate hepatocytes from liver pieces collected from other species with comparable sizes or comparable vasculature sizes. This may be important for researchers who use alternative models, such as porcine, canine or primate models.

Isolations of human hepatocytes are generally done using livers from two sources: whole livers deemed unsuitable for transplantation or morphologically normal liver tissue from resection margins. The advantage of the latter source used in this method is that the liver pieces are made available to the laboratory sooner. Immediately after resection, the resected liver is brought to a pathologist who removes what is required for diagnosis. The pathologist will then remove a suitable piece of morphologically normal liver for hepatocyte isolation from what is slated for discarding. In general, a liver piece arrives in the laboratory ready for perfusion in an average time of 56±29 min (N = 103). In comparison, whole livers that are not suitable for transplantation will only be released to the laboratory between 13±2 hr and 16±12 hr [15]. In addition, due to ethical considerations and the shortage of donor livers, isolation of hepatocytes from whole livers that do not meet all of the criteria for transplant is avoided in the Eurotransplant region. This is because these livers could still be used for transplantation with extended donor criteria. Some studies have shown that maximizing patient access to transplantation results in decreased wait list mortality and satisfactory outcomes to selected recipients [16,17]. Another advantage is that the cells obtained from liver resection pieces are isolated from morphologically healthy liver, while livers unsuitable for transplant can be steatotic, fibrotic or cirrhotic. As such, the method here results in a high yield of 13 ± 11 million hepatocytes per gram liver compared to the 0.7±0.3 million or 3±2 million hepatocytes per gram liver obtained by Baccarani, *et al.* [15] using cirrhotic or steatotic livers respectively. However, liver resection pieces result in a lower total yield of hepatocytes as the size of the piece available is generally small with a range from 2-250 g and an average of 37±29 g (N = 648).

In comparison to other groups, this protocol avoids the use of cyanoacrylate adhesives. Alexandre, *et al.* [18] found that the use of ethyl cyanoacrylate, a commonly used all-purpose adhesive, to cover the cut surfaces of the liver, results in an increased yield of hepatocytes

from 3.5±0.7 to 6.0±1.6 million hepatocytes per gram liver with values expressed in means ± standard error of mean. In comparison, this protocol is able to achieve a yield of 13.5±0.4 million hepatocytes per g liver (expressed in means ± standard error of mean) without the use of cyanoacrylate adhesives. While cyanoacrylate adhesives have been used for wound closure [19,20], medical grade cyanoacrylate adhesives such as octyl cyanoacrylate and butyl cyanoacrylate can be expensive compared to ethyl cyanoacrylate. However, shorter chain derivatives such as ethyl cyanoacrylate have been found to be more histotoxic than longer chain derivatives [21,22].

This method is simpler and more time efficient due to the use of curved irrigation cannulae with olive tips. The use of cannulae of an appropriate size will result in a tight fit that holds the cannulae in place without the use of glue [18] or sutures [23]. The cannulae used have diameters ranging from 1-2 mm with tip diameters sized from 1.25-4.5 mm. An assortment of various cannula sizes should be made available when a liver piece is ready for cannulation, so that the appropriate sized cannula for a particular blood vessel can be chosen as shown in the accompanying video. This method also utilizes more cannulae in general (**Figure 2A**) compared to other studies where 2 [23] or 2-4 [18] cannulae are used. This may help to achieve a better perfusion throughout the liver piece leading to high viability and yield in a shorter digestion period.

In conclusion, this protocol isolates human hepatocytes, which are an important model for studying cellular metabolism, pharmacokinetics and toxicology of xenobiotics, liver regeneration and translational research. A previous survey on 150 drugs that cause human toxicity showed that the concordance between toxicity found in animal studies and that observed in clinical practice is 70% [24]. Thus, human hepatocytes remain an important model for validating research done in animal models and for testing drug leads for adverse reactions before going to clinical trials. Further, the use of human hepatocytes isolated from remnant liver samples or hepatocytes isolated from slaughterhouse animals [25] as a model is in line with the 3R ethical framework [26] to replace the use of research animals when possible.

Disclosures

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