

## Review Article

# Recommended parameters for hepatocyte isolation-a review

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## ABSTRACT

There is an increasing demand for primary human hepatocytes for toxico-pharmacological studies and for exploring their role in liver cell transplantation. During the whole process of isolation, culture and cryopreservation hepatocytes are subjected to various stress. All these factors will lead to the impairment in the metabolic capability of the hepatocytes post isolation. Hepatocyte isolation is an expensive and resource driven procedure. Thus, it is important to consider all the factors before we subject the specimen for isolation of cells. By considering the factors that influence hepatocyte isolation, hepatocytes of good quality can be obtained. With recommendations on the ideal parameters for isolation being made the researcher shall have a knowledge on what critical factors need to be taken care of during hepatocyte isolation. There will be an opportunity to modify critical factors if possible, during the procurement of specimen by working in tandem with the clinicians. The specimen which shall be deemed un fit when two or more factors have exceeded the critical limits can be rejected.

**Keywords:** Hepatocytes, Culture, Isolation

## INTRODUCTION

The liver cells or primary human hepatocytes are isolated from liver surplus surgical specimen obtained after surgery.<sup>1,2</sup> Later they are either cultured or cryopreserved to be used on demand. Large quantities of primary human hepatocytes are required for basic research and transitional medicine especially in the field of pharmacotoxicology. Liver cell Transplantation and Liver dialysis could be the modality of treatment for acute liver failure in the days to come.

During the whole process of isolation, culture and cryopreservation hepatocytes are subjected to various stress.<sup>3</sup> Change in the microenvironment due to variations in temperature, exposure to various chemicals, hypoxia, lack of nutrients and accumulation of toxic metabolites

induces stress on the hepatocytes. Many demographic factors which also affect their isolation process are age, sex, primary disease of the liver, steatosis of the liver cells, cirrhosis of liver, hepatitis due to various causes. Other factors which are commonly implicated are the warm and cold ischemia, expanded criteria of liver donors like deceased cardiac donation, rejected liver for organ transplant.<sup>4,5</sup>

All these factors will lead to the impairment in the metabolic capability of the hepatocytes post isolation. This may also lead to increased cell breakage during culture and compromised synthetic function.

The commonest source for translational research is commercially available cryopreserved hepatocytes. Lack of the above-mentioned data make them unreliable to

predict the expected functionality. Also, they lack the reproducibility when subsequent batches (of the same company) are used for the same research. Although some prequalified hepatocytes are available, they are extremely expensive because they are subjected to various molecular test. These tests may sometimes not be the sole metabolic function needed in the proposed study.<sup>6,7</sup>

Hepatocyte isolation is an expensive and resource driven procedure. Thus, it is important to consider all the factors before we subject the specimen for isolation of cells.<sup>8</sup> We need to strike a fine balance between the cost of cell and tests for characterisation of the cells.<sup>9,10</sup>

Hence in this study we have to reviewed relevant literature and extrapolated the various critical ranges affecting the isolation process.

**FACTORS AFFECTING HEPATOCYTE ISOLATION**

**Table 1: Factors affecting hepatocyte isolation.**

Factors
<b>Donor demographic factors</b>
Age
Sex
Primary disease of liver
<b>Expanded donor criteria</b>
Serology (hepatitis B virus, hepatitis C virus, human immunodeficiency virus.)
<b>Intra operative factors</b>
Weight of liver
Cold ischemia
Warm ischemia
<b>Liver specific factors</b>
Steatosis
Cirrhosis
Cholestasis
<b>Isolation parameters</b>
Laboratory facilities
Media and Reagents

**Donor related factors**

*Age:* Older age leads to senility induced changes and the quality of hepatocytes tend to deteriorate. On the contrary the young age groups especially the neonate hepatocytes have very good metabolic and regenerative capabilities.<sup>11</sup> Wolfgang and their colleagues have demonstrated that any age above fifty years did not produce good yield and viability, similarly Serene Lee et al have also showed that hepatocytes from surgical specimens of patients aged above fifty years was also not promising.<sup>12</sup>

*Sex:* Sex of the donor influencing the character of hepatocytes isolated seems to be controversial. Ample literature exists for the increased incidence of acute liver failure in females. Females are more prone to acute liver

failure when exposed to toxic doses of hepatotoxic drugs to the tune of 1.5 to 1.7 times that of their male counterparts.<sup>13</sup> Mennecozzi et al have shown that even the isolated female hepatocytes are fragile leading to cell injury when exposed to hepatotoxic drugs.<sup>14</sup> Although exact reason for the same has neither been proven in clinical or experimental settings, with the available literature outcome seems to be good when the specimen is from a male patient.

*Primary disease of the liver:* Malignancy in general brings about cachexia changes in the human body. Exposure to chemotherapeutic drugs causes early changes in the liver in terms of increased cell lysis and fatty liver. Even late changes of liver are seen in the form of fibrosis or cirrhosis.<sup>15,16</sup> There are also chances of cholestasis in carcinoma of the biliary tract which further deteriorates the function of the hepatocytes. On the contrary benign lesions do not have such adverse impact on the quality of hepatocytes. Fan and his colleagues have shown favourable outcomes in hepatocytes isolated from specimens of benign liver disease.<sup>17</sup>

*Expanded criteria donor:* Due to various limitations in obtaining Surgical liver specimens, Research scholars and clinicians have always tried to alter the selection criteria. By expanding the donor pool, a greater number of livers are available but at the cost of inferior quality of hepatocytes. Expanded criteria donor includes deceased cardiac death donations, livers rejected for human organ transplant, remnants of split liver graft. The very fact that the whole organ is unsuitable for transplant implies that even cells are deemed to be of substandard quantity. The reasons may be multiple like excessive steatosis (>60%) fatty liver, early cirrhosis of liver, acute ischemic degeneration of hepatocytes owing to hypotension and prolonged ionotropic support.

Many attempts are made to optimise any adverse factors affecting hepatocyte isolation. Recent developments have shown recovery of hepatocytes after subjecting the organ to machine organ perfusion. This development has expanded the potential for more organ availability for cell isolation and also significantly improving the quality of the isolated cells as demonstrated by Maria-Louisa Izamis et al.<sup>18</sup> There are also reports of successful cell isolation even from rejected specimen, there is no absolute contraindication for cell isolation from rejected specimens.<sup>19</sup> However expanded criteria donor livers have shown to yield inferior quality cells. Hence this is also one detrimental factor.

*Serology:* Serology is defined as active or chronic hepatitis virus infection with B or C and human immunodeficiency virus. There are many other viruses that tend to cause in vivo hepatocyte lysis but these viruses are commonly encountered situations during surgical practice. These viruses are sometimes lodged into the hepatocyte or indirectly affect the hepatocytes. There is a lot of cell inflammation seen on histopathology

and biopsy in the early stages of the disease. Chronic phase shall always have some amount of liver cirrhosis radiologically or at least in histopathology. Keeping the following reasons in consideration a positive serology patient has some cell inflammation causing decrease in the quality of isolated cells.

### **Intra operative factors**

**Weight of the liver:** There are different modalities of liver cell isolation. The basic point of consideration is if there is an identifiable vasculature of appropriate size for cannulation. If the size of the vessel is adequate which we can cannulate, then 2 step or 3 step perfusion methods can be employed.<sup>20</sup> If the specimen size is small, then the specimen can be cut into small pieces and subjected to chemical dissociation.<sup>21</sup> In our experience, we have noticed that an approximate size of 25 gm shall have identifiable vessel that is the portal vein. In specimens less than 25 gm it is difficult to cannulate and further secure it for perfusion.

The size of the liver specimen is debated further and studies have shown that size does matters. In case of larger specimens' perfusion will be difficult at the level of capillaries as they will be collapsed and the perfusate will not be able to reach in adequate concentration. The hepatocytes which are dissociated from basement membrane cannot be easily freed and they will get entrapped in the scaffolds. Alexandre suggested that 100 gm as critical value for optimal cell isolation.<sup>22</sup> Serene lee has also given that, increasing the size of the liver, the concentration of collagenase needed to dissociate the cell also increases. We have kept 100 gm as cut off for ideal weight of liver for perfusion.

**Cold ischemia:** Cold preservation of the liver specimen tends to decrease the enzymatic activity of the cell and thereby the metabolic demand of the cell also decreases. Maintenance of predetermined cooling rate and consistent optimal temperature on cold storage is vital, failing which there will be excessive cell stress and breakage. In our study we have employed static cooled preservation technique that shall decrease the metabolic demand but shall not stop metabolism completely. Hence a critical time for safe preservation should be defined beyond which cell yield and viability will be decreased. Some authors have defined that there is no major influence on the isolation or viability if done with up to 24 hours of cold ischemia.<sup>23,24</sup> However practically in expanded pool liver donors this factor is of concern. In practicality of the clinical setup. The deceased donor liver shall be offered for human organ transplant and if deemed unfit shall be offered for experimental use. Lot of time is spent on the logistics and decision making by various transplant team. So, an extended criteria donor liver, exposed for prolonged ischemia is a bad combination. Further in liver surplus after hepatectomy specimens' lot of time is spent during transport from operation theatre to laboratory, especially when the liver explant is done during odd working hours. The dilemma of the optimal cold

ischemia time exists on what is the maximal cold ischemia time. Less than 24 hours of cold ischemia can be tolerated in normal livers.

**Warm ischemia time:** Warm ischemia is more detrimental to hepatocytes than cold ischemia as metabolic function is still present in normal temperatures, leading to cell energy depletion and death. The definition of warm ischemia also varies among different authors. The point of concern is whether the brief ischemia time between clamping of vessels to start of perfusion is included or not included. The other controversy is whether the warm ischemia is till the suspension of cells in wash solution during isolation or until the culture and cryopreservation media. Warm ischemia in our study is defined to include: The time of cross clamping of vasculature to start of cold perfusion, the time for cell isolation till they are suspended in final wash media.

Hence in our study warm ischemia includes both. Literature review shows an average of 60 minutes to be quite safe in preserving cells for isolation as shown by Izamis et al.<sup>24</sup> This 60 min is just upper limit of the available literature as many authors have defined less than 30 minutes as the safe period. Ambiguity still exists in the definition terminology.

### **Liver specific factors**

**Steatosis:** Steatosis or fatty liver is accumulation of fat in the liver which is a common scenario in the Indian population. The fat may be micro vesicular, macro vesicular, or mixed but the quantity of fat is more important. This is because excessive accumulation causes the cells to be fragile and will lyse during the process of cold ischemia, cell isolation, cryopreservation and thawing.<sup>26,27</sup> While authors have proposed different modalities of fat grading in the cells, we have used post-operative histopathology to grade the fat in the cells. The cells are divided as <30, 30-60 and >60%. This is the commonest terminology used by hepatologist, pathologist and transplant surgeons. Many studies have been used to correlate body mass index, steatosis and hepatocyte isolation. We have used histopathology as the criterion as many factors like diabetes, alcoholism, obesity, chemotherapy, non-alcoholic fatty liver disease etc will cause steatosis. In a publication elsewhere we have demonstrated steatosis above 30% affecting hepatocyte isolation keeping other confounding factors to the minimum. Alexandre et al has used 10% as the cut off, but this is difficult to quantify in regular clinical labs. Hence for the ease of reproducibility it is easy to use 30% as the limit.

**Cirrhosis:** Cirrhosis is the increased fibre content in the liver making the liver firmer. Cirrhosis is more detrimental than steatosis during cell isolation. Here the cells will be replaced by fibre tissue resulting in net loss of cells. We have not considered macro nodular or micro nodular cirrhosis separately. Cirrhosis on routine radiology is evaluated and specimens are graded on histopathology. Baccarani et al have not graded cirrhosis

separately but just noted the presence or absence of it.<sup>27</sup> If the cirrhosis is detected on histopathology after the explanation of liver, it is considered to be of milder degree and does not affect much in the process of hepatocyte isolation. If the specimen has cirrhosis on routine radiology like ultrasound scan, Computed Tomography Scan or Magnetic Resonance Imaging it indicates more pronounced form of cirrhosis. These severe form of cirrhosis shall seldom be encountered in liver resection or deceased donors. This severe form of cirrhosis will be seen in livers obtained from recipient liver in transplant. Hence it is quite prudent not to use liver which has cirrhosis on radiology examination.

**Cholestasis:** This is the obstruction of the normal biliary excretion leading to jaundice. The safe level below which a person can be subjected to liver surgery is a matter of debate and no consensus exists on this value. The toxic effect of bile stasis affecting the hepatocyte isolation has been described by Benjamin et al.<sup>29</sup> As the cut of value for cholestasis is still debatable, we have taken the minimum value as 1.5 mg/dl as the cut off for presence of cholestasis.

**Isolation parameters**

**Laboratory facility:** The presence of the laboratory in the same premises is considered ideal for conducting experimental research. This will help in getting better information from the clinicians about the liver. Further logistic issues, ischemia time and ethical issues are reduced and better addressed.

**Table 2: Recommended parameters for ideal isolation conditions.**

Donor demographic factors	Variables
Age	<60 years
Sex	Male
Primary disease of liver	Benign
Expanded donor criteria	Not recommended
Serology (hepatitis B virus, hepatitis C virus, human immunodeficiency virus.)	Negative
<b>Intra operative factors</b>	
Weight of liver	<100 gm
Cold ischemia	<24 hours
Warm ischemia	<60 minutes
<b>Liver specific factors</b>	
Steatosis.	<30%
Cirrhosis.	Nil
Cholestasis	<1 mg/dl
<b>Isolation factors</b>	
Laboratory facilities	In house
<b>Media and reagents</b>	Good
	manufacturing practice

**Media and reagents:** Many proprietary media are offered by different companies. Also, few kits are available

which have complete set of reagents along with their protocols. They are expensive and needs to be transported maintaining proper cold chain. Collagen is the only reagent which should be properly selected because degradation of collagen is the vital step in isolation of hepatocytes. Many types of collagen are used like collagen P, collagen IV, but the efficacy of one over the other is still not proven.<sup>30,31</sup>

Yield and viability are the two measurable parameters that shows the effectiveness of cell isolation procedure. Both of them vary greatly depending on the above-mentioned factors. The obtained cell solution is processed in Neubauer counting chamber using trypan blue staining as routine by many authors. Some authors have used specialised automated cell counters.

**Viability:** This is expressed in percentile and is nothing but the percentage of viable cells in the solution. The below mentioned formulae is used for counting.

Viability (in%) =total number of viable cells in 1 mm<sup>2</sup>/total number of cells counted in 1 mm<sup>2</sup> X 100.

**Yield:** Yield of the tissue is defined as the number of cells retrieved per gram of liver tissue. This is measured in terms of millions per gram of liver tissue.

Yield (million hepatocytes/ml of cold storage solution) =average number of cells in 1 mm<sup>2</sup> x trypan blue dilution factor x haemocytometer factor.

(Trypan blue dilution factor is 10 and Haemocytometer factor is 10,000 and these factors depend on dilution factors)

Yield (million hepatocytes/grams of liver) =yield (million hepatocytes/ml of cold storage solution) x Total volume of resuspended cells/Weight of liver in grams.

Primary human hepatocytes are still considered as the gold standard in many experimental research studies. Hepatocyte spheroids and 3D cultivation have shown a promising result for the evaluation of drug toxicity.<sup>33</sup> The main drawback is the availability of prequalified hepatocytes on demand and the cost of procurement. This can be overcome by having isolation facility in the research laboratory.

Hepatocytes can be procured as liver surplus tissue after hepatic surgeries. But care should be taken for proper selection of the specimens. The above-mentioned factors or variables shall greatly enhance both yield and viability.

Many commercially available proprietary media shall give good yield and viability and bring the cost down as compared to the commercially available hepatocytes. Inhouse facility shall greatly enhance the interaction between the clinicians and research scholars which is necessary to consider the factors discussed as above.

With such recommendations on the ideal parameters for isolation the researcher shall have a knowledge on what critical factors need to be taken care of during hepatocyte isolation. There will be an opportunity to modify critical factors if possible, during the procurement of specimen by working in tandem with the clinicians. The specimen which shall be deemed un fit when two or more factors have exceeded the critical limits can be rejected. As per our knowledge and review of literature there are only a few studies which have worked on clinical, demographic and laboratory data in cohesion.

## CONCLUSION

Hepatocyte isolation is a resource demanding procedure. Proper selection of liver tissue is essential. By considering the factors that influence hepatocyte isolation, hepatocytes of good quality can be obtained.

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