

## Original Research Article

# Assessment of albumin and aspartate levels as a simple indicator of the efficacy of cryopreservation

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## ABSTRACT

**Background:** The process of hepatocytes cryopreservation is standardised by most of the laboratories. However there is a variation with respect to the Protocols, media and equipments used amongst the laboratories. Similarly, the tests available to evaluate the efficacy also varies. They are expensive and sometimes might not measure the parameter required for a particular research study. Hence we propose a methodology to study the few basic parameters like cell viability, synthetic function of the cell and cell stability. We have also used a simple percentile calculation to know the efficacy of cryopreservation. This shall help in functional validation of the cell after cryopreservation. The same can also be used to compare the quality of hepatocytes between different batches. The objective of the study was to characterisation of the cells to determine the efficacy of cryopreservation.

**Methods:** Two step collagen isolation method was used to isolate the hepatocytes. Initial cell viability was calculated. A sample of cells were taken for characterisation and the remaining cells cryopreserved. The sample cells were divided into two batches one for pre cryopreservation culture and the other for post cryopreservation. The pre cryopreservation culture was done on monolayer using collagen coated 6 well plate. The other sample was placed in the cryovials for cryopreservation for 1week. After 1 week the cryopreserved cells were thawed and the post cryopreservation viability calculated, followed by post cryopreservation culture. During the process of culture (both pre cryopreservation and post cryopreservation) for 5days Albumin was measured daily and average calculated, peak Aspartate (AST) at 24 hours was recorded. The percentile difference of the obtained values between the pre cryopreservation and post cryopreservation culture was calculated.

**Results:** A total of 12 specimen were enrolled for the study. The mean pre cryopreservation viability of the cells was 66.58%. The post cryopreservation, viability of the cells was 36.43%. The mean difference was -30.170%. The pre cryopreservation albumin values had a mean of 150ng/ml. The post cryopreservation albumin values had a mean of 135.83ng/ml. The mean difference was -14.170ng/ml. The pre cryopreservation peak Aspartate values had a mean of 234.17 IU/ml. The post cryopreservation peak aspartate values had a mean of 230 IU/ml. The mean difference was -4.176 IU/ml.

**Conclusions:** This simple method can validate the cells after cryopreservation by measurement of cell viability, synthetic function of the cell and cell stability.

**Keywords:** Albumin, Cryopreservation, Culture

## INTRODUCTION

Human hepatocytes are still considered as gold standard for research studies. But the main drawback is it is

expensive and sometimes not available on demand. Hence many laboratories have resorted to store the hepatocytes by cryopreservation for future use. But the deterrent is that the cryopreserved hepatocytes may not

optimally function after cryopreservation. Thus, we need a validation process to know the impact of cryopreservation and their profile after recovery.

Cryopreservation is the process of cooling and storing the cells, at very low temperatures to maintain their viability for use in the future.

The cells are subjected to lot of stress during the process of cryopreservation and thawing. This will have an impact on the post thaw viability, plating efficacy and metabolic function. Suggesting that cryopreserved hepatocytes may not function optimally if the cryopreservation is not done properly. Thus, efficient cryopreservation methods shall give sustained supply of good quality of hepatocytes for the purpose of research.<sup>1,2</sup>

Lot of protocols have evolved in the recent past for effective cryopreservation, Cytoprotectants being one of them. Also, various proprietary media have been developed which are commercially available.<sup>3</sup> Lot of challenges are present in large scale cryopreservation of hepatocytes, maintaining core temperature being the most important. Novel methods of vitrification and encapsulation are also being tried. These novel methods are widely used in clinical therapeutics especially liver cell transplant.<sup>4,5</sup>

All the above methods have helped in the efficient long term and large-scale cryopreservation of hepatocyte. But the gist of the problem which needs to be addressed is the ability of the cell to recuperate to the original metabolic activity after the stress during the cryopreservation and thawing.

As of now we have many methods to validate the preserved function of cell after cryopreservation. Few of the frequently measured parameters are attachment efficiency, albumin and ureogenic capability, phase I and II enzymes activities and the expression of specific adhesion molecules in vitro. These tests are expensive and requires sophisticated equipment. These tests may not be the functional parameters that is required for translational research studies.<sup>6</sup>

Further in laboratories where the isolation and cryopreservation facility are present the quality assessment is necessary and they need to compare the cells between batches. A simple preliminary sampling method of the whole lot of cells will benefit before subjecting them to complex and expensive steps. This process should be simple and less time consuming (duration of cryopreservation). The validation tests should not require sophisticated instruments and imported kits.

Thus, we have proposed a simple test that addresses issues like viability of the cell, their synthetic function and stability after cryopreservation.

## METHODS

Ethical clearance was obtained from the institutional ethical committee. Informed written consent was taken from the participating patients before the surgery.

Patients with cirrhosis, steatosis, and age above 60% was excluded from the study. Initial yield of less than 1 million per gram of liver and viability of less than 50% was excluded from the study.

Liver tissues were obtained as surplus after resection surgeries. Liver tissues with veins broad enough to cannulate were subjected to a two-step collagenase isolation process.<sup>7</sup> The specimen which were small and no identifiable veins were subjected to direct chemical dissociation.<sup>8</sup>

Following the process of isolation, towards the end of the procedure, a sample from the isolated lot was procured. The main set of cells were subjected for further cryopreservation.

Trypan blue test was done on the sample batch, and the concentration of that particular batch was determined. The sample cells were adjusted to 2-3million cells/ml and divided into two batches. First batch was subjected to pre cryopreservation culture and the second one for cryopreservation and subsequently Post cryopreservation culture.

The protocol for pre cryopreservation culture and the post cryopreservation culture remained the same. The values were entered in the predetermined proforma and the following parameters were measured i.e. viability, average albumin production for 5 days and peak AST (aspartate alanine transferase) after 24 hours.

All the above 3 values were measured for pre cryopreservation and post cryopreservation.

We have used the 6 well collagen type 1 coated plates with 9.6 surface area (cm<sup>2</sup>) and working volume of 1-3ml and seeding density of 0.3×10<sup>6</sup> cells. 2.5 ml of the hepatocyte plating media was inoculated into each well. The plate was covered with a lid and transferred from the hood to the incubator.

The cells were incubated for 3-4 hrs in 5% CO<sub>2</sub>, 37 0 C and 80-90% humidity. After 3-4 hrs, the plates were removed and placed back into the hood. About 2ml plating solution was aspirated and replaced with 2 ml of hepatocyte maintenance media. After replacement of the fluid the plate was transferred back into the hood and incubated for 24 hrs with the same setting of incubator (5% CO<sub>2</sub>, 370C and 80-90% humidity).

At the end of every 24 hours the supernatant of approximately 1-1.5 ml from each well was aspirated and collected. Each well was then replaced with 1-1.5 ml of hepatocyte maintenance media and the culture plate was

placed back into the incubator with the same settings. All the supernatant aspirate was collected in a 10ml test tube and sent for analysis.

The supernatant aspirate was used to analyse the concentrate of Albumin and Aspartate Aminotransferase. Albumin secretion was measured for 5 days and entered into the proforma. The average value of the albumin secretion was taken. The level of Aspartate Aminotransferase (AST): which peaks in the first 24 hours was taken and entered in the proforma.

The second sample was subjected for cryopreservation. Media exchange was done, and the cells were suspended into the cryopreservation media and the final concentration was adjusted to approximately 2 - 3million cells/ml. The pre cryopreservation viability was then determined. The contents were placed in the cryovials for further cryopreservation. The cell suspension was labelled as Cryopreservation-1week.

The cryovials were placed back into Mr. Frosty™ Freezing Container. The cryovials and Mr Frosty were maintained in the -800 c freezer overnight. This gives a stable decrease in temperature. After overnight storage of cryovials, they were removed from the Mr Frosty and transferred into the liquid nitrogen below -135°C.

Protocols were fixed for the process of thawing and plating. The thawing and plating media were pre warmed to 37°C in a water bath. The cryopreserved hepatocytes were then thawed in 37°C water bath for <2 min. Using a wide-bore pipette tip the hepatocytes from the cryovial were transferred into the thawing media (15ml). The contents were gently mixed and Centrifuged at 100 x g for 10 min at 40c. The supernatant fluid was discarded 10 .The same standard counting methods as described in the previous section was used to determine the number of viable cells/ml of fluid. This gave the post thaw viability count. The sample was subjected to post cryopreservation culture and Albumin and AST values were determined.

### Formulae

Viability: (Pre cryopreservation viability - Post cryopreservation viability)/Post cryopreservation viability ×100.

Albumin: (Post cryopreservation albumin average-Pre cryopreservation Albumin average)/Post cryopreservation Albumin average ×100.

Aspartate Alanine Transferase AST: (Post cryopreservation AST-Pre cryopreservation AST) / Post cryopreservation AST ×100.

### RESULTS

A total of 19 specimens were included in the study during the process of isolation. But due to the exclusion criteria as mentioned above only 12 specimens (n=12) were considered for cryopreservation.

In the study group we had 5 females and 7 male patients. Average age was 42.92 years with an interquartile range between 20 years to 60 years.

The mean pre cryopreservation viability was 66.58 and mean post cryopreservation viability was 36.42 with a mean difference of -30.170, and this was statistically significant with a p value of 0.000 (p value <0.001).

The mean of pre cryopreservation average albumin was 150.00 and the mean of Post cryopreservation average albumin was 135.83. The mean difference was -14.170. The groups on comparison were not significant statistically (p=0.040).

The mean of pre cryopreservation peak AST was 234.17 and the mean of post cryopreservation peak AST was 230.00. The mean difference was -4.167. The groups on comparison were statistically not significant with a p value of 0.636 (p<0.001).

**Table 1: Comparison of pre cryopreservation and post cryopreservation viability.**

Specimen number	Pre cryo viability	Post cryo viability	Difference	% decline / raise
1.	60	42	-18	-30
2.	75	40	-35	-47
3.	69	38	-31	-45
4.	56	35	-21	-38
5.	75	45	-30	-40
6.	67	30	-37	-55
7.	70	42	-28	-40
8.	67	39	-28	-42
9.	74	40	-34	-46
10.	63	30	-33	-52
11.	64	35	-29	-45
12.	59	21	-38	-64
<b>Mean</b>	66.58	36.43	Mean difference -30.170	
<b>Std deviation</b>	6.374	6.708		
<b>T value -30.170</b>				P value 0.000

**Table 2: Comparison of pre cryopreservation and post cryopreservation levels of albumin.**

Specimen number	Pre cryo Albumin ng/ml	Post cryo Albumin ng/ml	Difference	% Decline / Raise
1.	150	120	-30	-20
2.	100	60	-40	-40
3.	120	100	-20	-17
4.	200	160	-40	-20
5.	210	200	-10	-5
6.	110	90	-20	-18
7.	140	110	-30	-21
8.	150	170	20	13
9.	170	180	10	6
10.	150	140	-10	-7
11.	200	180	-20	-10
12.	100	120	20	20
<b>Mean</b>	150	135.83	Mean difference -14.170	
<b>Std Deviation</b>	38.847	42.738		
<b>T value 2.327</b>				P Value 0.040

**Table 3: Comparison of pre cryopreservation and post cryopreservation peak AST.**

Specimen number	Pre cryo AST IU/ml	Post cryo AST IU/ml	Difference	% Decline / raise
1.	250	280	30	12
2.	270	250	-20	-7
3.	230	270	40	17
4.	180	160	-20	-11
5.	190	140	-50	-26
6.	270	300	30	11
7.	280	310	30	11
8.	210	200	-10	-5
9.	200	180	-20	-10
10.	260	220	-40	-15
11.	180	170	-10	-6
12.	290	280	-10	-3
<b>Mean</b>	234.17	230	Mean difference -4.176	
<b>Std Deviation</b>	40.778	59.084		
<b>T value 0.486</b>				P Value 0.636

## DISCUSSION

Hepatocytes are required in large scale for biomedical research like bioprinting, liver dialysis, artificial organs. These essentially rely primarily on cryopreserved hepatocytes. Cryopreserved hepatocytes have shown to be better than freshly isolated hepatocytes in terms of storage, ease in transportation, availability on demand. But the major challenge is in preservation of metabolic function and achieving adequate post cryopreservation viability. Newer methods to bridge this gap have been evolving.

The main concept of cryopreservation is to preserve cellular and subcellular structure like nucleic acid and proteins at optimal temperature and storage conditions for preserved activity. Water which is the major component of life when frozen ceases all the metabolic activity.

Many factors influence cryopreservation of cells like cell volumes, optimisation of cooling rates, storage condition temperature, rewarming rates, cryopreservation media, cytoprotectants, damage to cellular and sub cellular structures.

Most common damage in the sub cellular level is mitochondrial membrane potential (MMP), a decrease in adenosine triphosphate (ATP), and loss of iron, free radical injury.<sup>11</sup>

In our study, the mean age was 42 years, patients above 60 years were excluded from the study owing to the excessive friability of the cells.<sup>12</sup> Patients with excessive steatosis and proven cirrhosis have shown to have decreased yield and viability.<sup>13</sup> We have not used cells with the initial yield less than 1million per gram and the cell viability of less than 50%. Since we have not used

precoll method to purify the cells we have excluded inferior quality liver tissue and cells.

In our study we have analysed the cells under the following parameters 1. Viability of the cells after cryopreservation. 2. Synthetic function of the cell by measuring the albumin. 3. Peak AST level to know the stability of cells in the culture conditions.

Viability of the cell is calculated in percentage in respect to the number of live cells to the total number of cells counted. There is always a drop in the post cryopreservation viability. The mean difference was -30.170 and the difference between both the groups statistically significant (p value 0.000).

This difference is highly variable, and it depends on number of demographic variables also. Alexandre in 2002 has demonstrated that recovery of hepatocytes depends on various factors.<sup>12</sup> A drop of nearly 50% was seen in few cases in similar studies. Hence this parameter was used in our study. The measurement of this parameter can be done by a simple Neubauer counting chamber or an automated cell counter. Simple stains such as trypan blue shall serve the purpose.

After comparing the viability we have also cultured the cells to know the synthetic function of the cell and stability of the cells on culture.

We have not tried to analyse the plating efficacy in the study because they depend on number of parameters like-collagen coated wells, micro circulated wells, ultra-low attachment plates for spheroid etc. Likewise, the medias used are commercially proprietary medias. There are no studies proving the superiority of one media over the other. Hence, we recommend using any media as per the protocol followed routinely in the laboratory.

There are many advanced culture systems including the perfusion based, 3D culture systems, 3 dimensional Spheroids. In our study we have used the basic monolayer culture on collagen 1 coated plates. This is based on the simple assumption that, if the cell performance of metabolic or sustainability is good in the basic system then it will perform aptly in all the advanced culture systems.<sup>15,16</sup>

Albumin is secreted exclusively by the liver. This is one of the vital elements in blood involved in transportation and in maintenance of intra vascular osmotic pressure. This signature secretion is measured in our culture.<sup>17</sup> By measuring the albumin we have tried to know the synthetic capability of the cell. We have observed that albumin secretion was low on first day later rises on 2<sup>nd</sup> and 3<sup>rd</sup> day and after 5 -7 days it shows a decline. Hence with this observation we have used an average of 5 days of albumin secretion.

This study has compared the percentile difference between pre cryopreservation and post cryopreservation. We have seen a mean difference of -14.170 with a p value of 0.040. This suggests that there is no major difference between the groups. This implies that the secretory function of the cells is well preserved.

The sheer stress on the isolated cells will cause the cells to break down during the cryopreservation or during the culture. This will release lot of intracellular enzymes into the extracellular space. By quantifying this we shall be able to know the cell death in the medium. Aspartate transaminase (AST) or aspartate aminotransferase, also known as AspAT/ASAT/AAT or (serum) glutamic oxaloacetic transaminase (GOT, SGOT) is commonly measured in all the standard liver function tests. This has been shown to be a reliable marker of hepatocyte breakdown since its introduction by Arthur Karmen and colleagues in 1954.<sup>18</sup> Alanine transaminase (ALT) is a similar marker commonly used, mostly used in conjunction. Due to similarity in function we have measured only one marker.

AST will show a peak raise in first 24 hours of culture and shall always show a decrease. Therefore, we intend to measure the peak AST. The difference in the increase or decrease in the AST values and the calculation of the percentile of increase or decrease has also been described above. An increase in the post cryopreservation value suggests that a greater number of cells are being broken down. Hence a negative value on percentile calculation suggests that lesser cells are being destructed.

On comparison between the groups we have observed a mean difference of -4.176 and a p value 0.636. The group had no statistical difference between them. By this we have can conclude more cells have not got destructed after cryopreservation and culture.

In summary of the above discussion we have used simple methods and calculation to validate the efficacy of cryopreservation and the subsequent behaviour of cells on culture. This simple validation can also be used to know the trend of cells of isolation, cryopreservation and culture in the laboratory. Further this can be used to compare the results between protocols. Researcher will have a prior knowledge of the behaviour of cells before subjecting them to expensive experiments.

There is no consensus on the cell validation as per our literature survey as there are no recommended cut off values for use or disuse the cells. The validation process at present rely only on local laboratory protocols. Similarly, we have not recommended any cut off values. These values are merely observations and greatly vary based on the cryopreservation and culture methodology and medias used.



## CONCLUSION

This simple method of assessment of levels of albumin and ALT from the culture supernatant can validate the cells after cryopreservation by measuring the cell viability, synthetic function and its stability.

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