CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2012 Vol. 1 (2-3) Jul-Sept. & Oct.-Dec., pp.64-68/Deshmukh
Research Article

RAT HEPATOCYTES MAINTAINED AT A REFRIGERATING TEMPERATURE FOR ONE WEEK EXHIBIT A HIGH DEGREE OF VIABILITY

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ABSTRACT
Cryopreservation of cells and cell lines is of immense importance, for use in future. In addition, it is a prerequisite for transportation of cells from one lab to another, from the clinic to the cell culture lab where the cells are propagated for diagnostic and therapeutic purpose and then from lab to the clinic for therapeutic purpose. The cells are routinely transported either through dry ice or liquid nitrogen. However, freezing and thawing of cells result in the loss of their viability to a significant extent in spite of having cryoprotectants like DMSO/Glycerol in them. In addition, the time duration of transport also matters the most. Due to technical clearance at the airports, transportation offices and the route of transport, the time lapse is inevitable. This again results in loss of viability of cells. In our laboratory, we have consistently observed that isolated rat hepatocytes when stored at 4^{o}C for seven days exhibited 83-85% viability. The viability testing was carried out by bright field microscopy using trypan blue dye exclusion methodandby fluorescence microscopy using calcein-AM Propidium iodide method. Since the storage was at 4^{o}C, there was no formation of cytoplasmic ice crystals. This is a simple approach to preservethe isolated hepatocytes above the freezing point of water for a period of seven days.

Keywords: *Cryopreservation, Animal Cell Culture, Viability, Cryoprotectants, Dye Exclusion Method, Calcein-AM Propidium Iodide Assay*

INTRODUCTION
Primary cell culture is a technique of growing the cells from the host which may be a patient or a healthy individual or an experimental animal. This is routinely carried for growing the cells from tumor, for diagnosis and research (Patel *et al.,* 2000). Culturing the amniotic cells (Gosden, 1983; Hoo *et al.,* 1983), chorionic villus sampling (Heckerling and Verp, 1991) and product of conception are employed in chromosomal studies and genetic diagnosis. These samples are obtained in the clinic or operation theatre, whereas culturing of the cells is carried out in animal cell culture laboratory, which is not necessarily situated in the same clinic or city. Therefore, the sample has to be transported to the location where the cell culture laboratory is available. As soon as a piece of tissue is excised it is cut off from the blood supply. As a consequence, the cells from the tissue experience stressful conditions such as anoxia, lack of nutrients, accumulation of metabolic waste, etc. Therefore, the tissue is preserved to a lower temperature to cease its metabolic activities in dry ice or liquid nitrogen cans. In order to prevent injury to the tissue or cells due to freezing, cryoprotectants such as glycerol 5-20% v/v and dimethyl sulfoxide (DMSO) 5-15% v/v are routinely used. However, glycerol and DMSO cause osmotic stress (Hak *et al.,* 1973; Armitage and Mazur, 1984). Moreover, there is significant decrease in viability of cryopreserved cells after thawing (Heng *et al.,* 2006). In the present investigations, the hepatocytes isolated from rat liver were stored at 4^{o}C for seven days and used for the study of Viability.

MATERIALS AND METHODS
Isolation of Hepatocytes from Rat Liver: Laboratory rat *Rattus norvegicus* was used with due permission of Institutional animal ethics committee. Rat of age three months was sacrificed under deep anaesthesia using diethyl ether. The liver was excised under aseptic conditions in the laminar airflow in sterile phosphate buffered saline. The liver was minced to generate fine pieces, which were repeatedly washed to remove traces of blood. The minced pieces were transferred to a sterile 15ml Falcon tube and treated with

3 ml of 0.25% trypsin containing 0.02% EDTA and 0.08% collagenase type I for 20 min at 37^{o}C. The trypsin and collagenase activity were inhibited by addition of 200µl of fetal bovine serum and centrifuged at 800 RPM. The supernatant was aspirated using a sterile transfer pipette and the enzyme treated pieces of liver were resuspended in Dulbecco's minimal essential medium supplemented with 10% FBS, 200mM L glutamine, 10mM HEPES and gentamycin 50µg/ml. The suspension was vigorously triturated to disaggregate the cells. Finally, the cell suspension was filtered through sterile 40µ cell strainer in the sterile Falcon tube. The filtrate containing isolated hepatocytes was used for viable cell count. The aliquots of cell suspension were stored in a refrigerator at 4^{o}C and viability was carried out after seven days. Each trial was carried out sextuplicate.

Viable Cell Count Using Trypan Blue Dye Exclusion Method (Strober, 2001): 50µl cell suspension was mixed with 50µl of 0.4% trypan blue stain. The sample was loaded in Neubauer chamber and viable cell counting was carried out. Since plasma membrane of viable cellsis impermeable to trypan blue molecules, live cells appear colourless, whereas plasma membrane ofdead cells loses its selective permeability, the dye molecules enter the cells, and therefore, dead cells appear blue. The percent viability was calculated using the formula:

% Viability= No. of unstained cells/total no. of cells X 100

Viable Cell Count by Calcein- AM Propidium Iodide Assay (Jones and Senft, 1985; Bratosin et al., 2005): Calcein AM is a non-fluorescent, hydrophobic compound that easily enters the intact, live cells. The hydrolysis of Calcein-AM by intracellular esterases produces Calcein, a hydrophilic, strongly fluorescent compound that gets excited at 495nm and emits a light of 515nm. Calcein is well-retained in the cell cytoplasm and therefore, live cells exhibit green fluorescence.

The damaged membranes of the dead cells allow propidium iodide to enter the cells, and the dye molecules bindto DNA by intercalating between the nitrogen bases. Thus, dead cells emit red fluorescence. 2 µl of Calcein-AM and 1 µl of Propidium iodide were added into 1ml of isolated hepatocytes suspension. The sample was vortexed and incubated at 37^{o}C for 5 min. The sample was loaded on sterile Neubauer chamber and cells emitting green fluorescence (live cells) and red fluorescence (dead cells) were counted.

RESULTS AND DISCUSSION:

The percent viability of isolated hepatocytes is enlisted in table 1. The appearance of isolated hepatocytes by Trypan blue dye exclusion method is in Figure 1A and Calcein –AM Propidium iodide assay in figure 1B. In fresh sample,by trypan blue dye exclusion method,it was 94$\pm$4% and infridge stored sample after seven days it was85$\pm$5%. In fresh sample by calcein-AM: Propidium iodide assay, 93$\pm$6% hepatocytes were viable; whereas, fridge stored sample had83$\pm$7% viable hepatocytes. There was decrease in % viability in hepatocytes suspension stored at 4^{0}C as compared to the fresh sample. However, this decrease was statistically insignificant. The morphology of hepatocytes at 1000X magnification is shown in Figure 2C and D. There was no any morphological variation in fridge stored samples as compared to fresh samples.

Since, the hepatocytes were stored at refrigerating temperature i.e. at 4^{o}C there was no any freezing related injury to the hepatocytes.

During cryopreservation and thawing the damage to the cells is caused by mechanical injury by ice crystals, concentration of electrolytes, dehydration of the cells and alteration in the pH (Coriell, 1979). The ice crystals cause damage to membranes and cell organelles which is the major contributing factor in cell death during cryopreservation Mazur (1984). Therefore, cryopreservation usually results in low cell recovery. Dietmar *et al.,* (1992) demonstrated 22% viability of cryopreserved rat hepatocytes. Zaleski *et al.,* (1993) found 42% viability of rat hepatocytes which were cryopreserved at -196^{0}C in liquid nitrogen. Swales and Utesch (1998) observed 85 $\pm$ 9% viability of cryopreserved dog hepatocytes suspension. Miyamoto *et al.,* (2006) demonstrated that cryopreserved rat and human hepatocytes supplemented with di-, tri-, and tetra saccharides in the culture medium exhibited significantly higher viability after thawing as compared to control.

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Table 1: Percent Viability of Rat Hepatocytes by Trypan Blue Dye Exclusion Method and Calcein-Am Propidium Iodide Method

Staining Method	% Viability of Freshly Isolated Rat Hepatocytes	% Viability Fridge Stored Rat Hepatocytes
0.4 % Trypan blue stain	94± 4	85± 5^{NS}
Calcein-AM Propidium iodide	93± 6	83± 7^{NS}

NS indicates statistically nonsignificant p>0.05

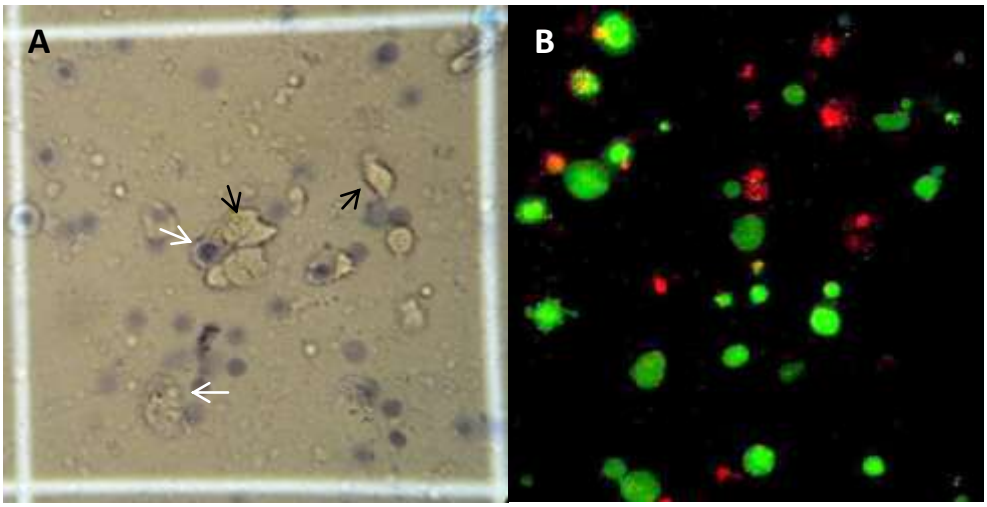


Figure 1: 400X Magnification Images of Viability Testing of Hepatocytes A) Trypan Blue Dye Exclusion Method: Black Arrow Indicates Unstained (Live) Cell, White Arrow Indicates Stained (Dead) Cell B) Calcein-AM Propidium iodide Method: Green Fluorescence Indicates Esterase Activity in Viable Cells, Red Fluorescence of Propidium Iodide –DNA Adduct Indicates Dead Cells

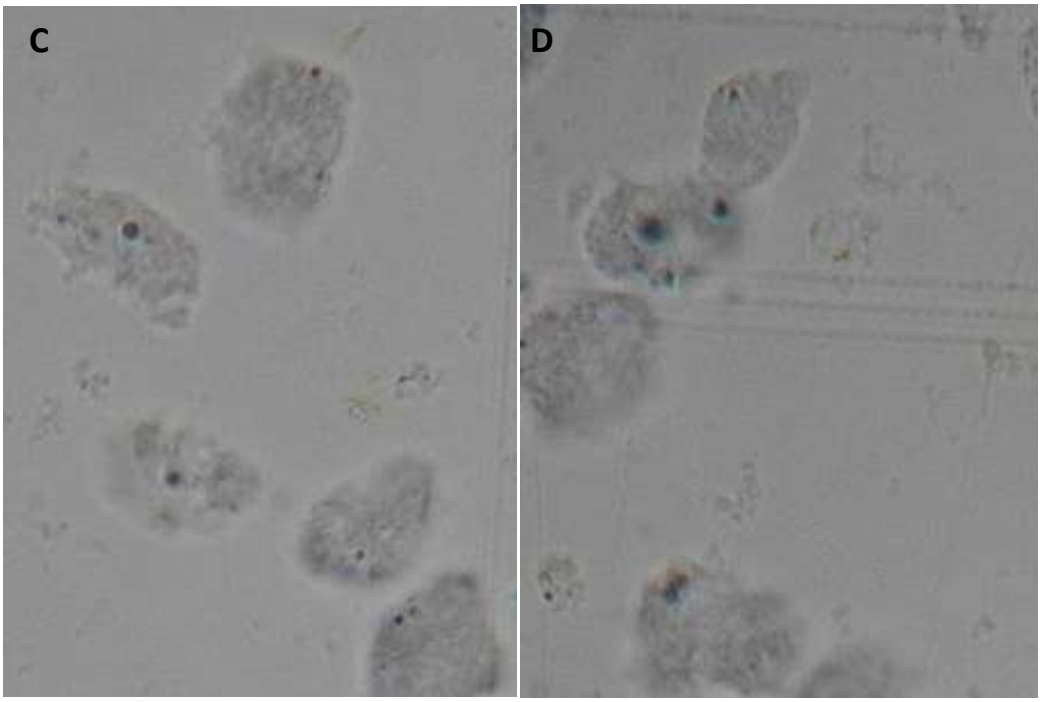
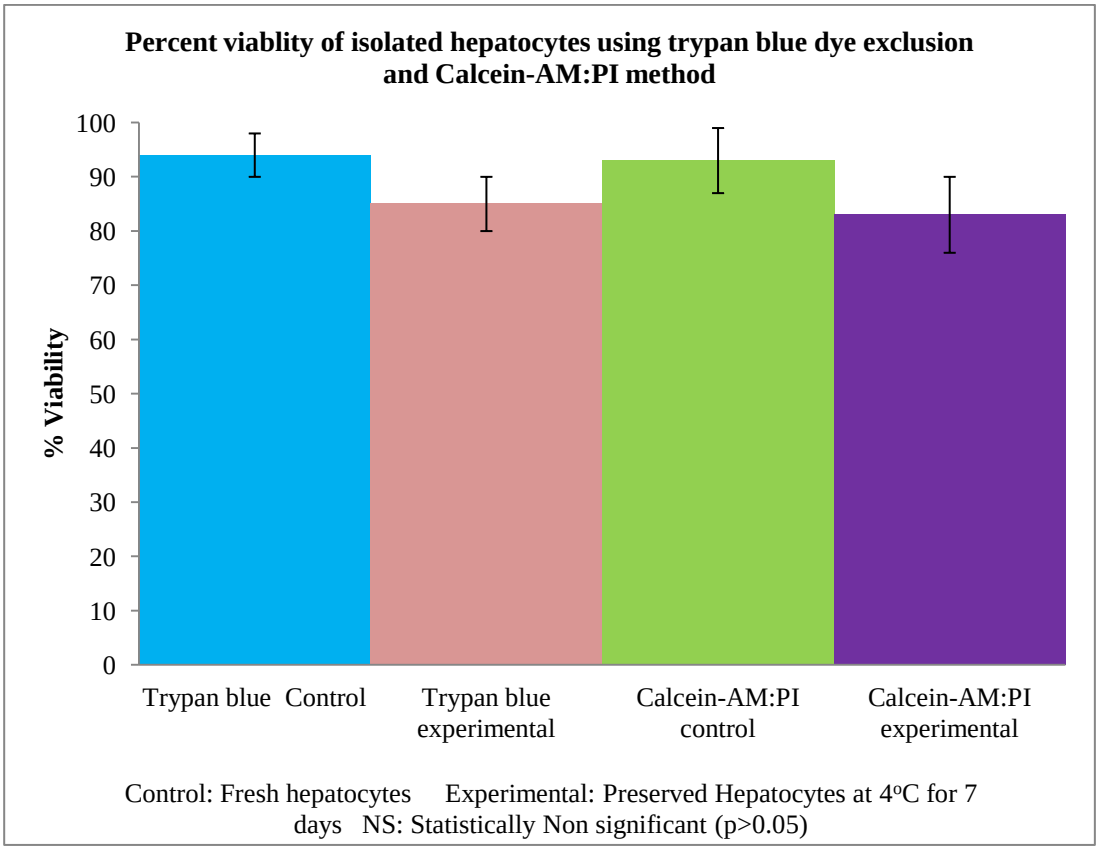


Figure 2: Shows Morphology of Rat Hepatocytes at 1000X Magnification; C) Fresh Hapatocytes D) Fridge Strored Hepatocytes after Seven Days at 4°C



The present findings provide convenient and economic way of storage and transportation of cell samples without compromising the cell viability. In addition, it will help teachers involved in conducting the training of primary cell culture in their teaching program. This is because; it is need of the hour to use a sacrificed experimental animal very efficiently by using as many organs as possible for primary culture. However, for use of fridge stored hepatocytes for experimentation as cellular models, additional studies about attachment and survival rates in cell culture flasks, metabolic characterization are needed.

Conclusion

The present study provides the simplistic and economic approach of preservation of disaggregated hepatocytes suspension to 4°C yielding higher viability than liquid nitrogen cryopreserved samples documented earlier.

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