Establishing an Optimised Protocol for Cryopreservation of Hepatocyte Microbeads for Clinical Use

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Establishing an Optimised Protocol for Cryopreservation of Hepatocyte Microbeads for Clinical Use

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Abstract

Acute liver failure (ALF) carries a high mortality, especially for neonates and young children, mainly because of the scarcity of timely suitable donor organs. At King's College Hospital, human hepatocytes transplantation has been used as an alternative technique to liver transplantation to treat children with ALF, were human hepatocytes isolated from unused or rejected donor livers (fresh or cryopreserved) are encapsulated in immunologically inert alginate forming hepatocyte microbeads (HMBs). The HMBs are then transplanted intraperitoneally under ultrasound guidance. The encapsulated-hepatocytes support the failing liver by providing the missing liver-specific functions, either until a suitable donor liver tissue is available for transplantation, or due to the host liver regenerative potential may lead to patient fully recovery without the need for liver transplantation. Dr. Mitry at King's established an optimised protocol for the production of clinical grade HMBs, and Dhawan and colleagues (2020) reported the safety and feasibility of intraperitoneal transplantation of clinical grade HMBs in children with ALF without the need for immunosuppression, were some patients recovered without the need for liver transplant. Alginate encapsulation allows nutrients, and oxygen diffuse in, and vital proteins and waste products diffuse out. The alginate coating does not allow antibodies, complement or immune cells to enter the microbeads, thus protecting the liver cells from the host immune response without the need for immunosuppression.

The main aim of this research project was to establish an optimised protocol for cryopreservation and future banking of hepatocyte microbeads making them readily available for acute liver failure emergency cases.

Human and rat hepatocytes were isolated using a standard collagenase perfusion technique, and cryopreserved (cell suspension or HMBs) using a controlled-rate freezer. Also,

vitrification method was tested for HMBs cryopreservation. Encapsulation of hepatocytes was carried out with GMP grade (Alginate SLG-20), and Inotech® R-50 Encapsulator. Seven different commercially available ready-made cryopreservation solutions tested were: TC-Protector, Cell Banker1, CP1, Cryo-JIN, NutriFreez™D10, pZever and University of Wisconsin (UW) solution, were used for cryopreservation of hepatocytes, and HMBs. Moreover, UW solution supplemented with various cryoprotectants; 0.2M Trehalose, 60µM Z-VAD, 2% PVP and 1.5M ETG were tested. After thawing, all hepatocytes, and HMBs (human and rat) batches were cultured, and hepatocyte-specific functions and activity were assessed using MTT Assay (cell overall activity), urea synthesis (ammonia detoxification), albumin synthesis (ELISA), EROD assay (cytochrome P450 activity; drug metabolism), and cell viability using FDA/PI staining.

Results of the tested readymade cryopreservation solutions showed that other than UW, Cryo-JIN and Cell Banker1 solutions could be used to cryopreserve hepatocytes and HMBs, these solutions maintain good cell attachment, overall activity, ammonia detoxification, albumin synthesis and drug metabolism functions.

After testing the seven readymade cryopreservation solutions, more optimization was conducted using UW solution. Eight different cryopreservation conditions with different types and concentration of cryoprotectants were used to test their efficiency in hepatocyte cryopreservation, each contain different type of cryoprotectants (CPAs) and compared with Condition 1 (UW + 5% glucose + 10% DMSO). The results showed that addition of Z-VAD alone and/ or Trehalose to UW solution improved the cryopreservation outcome of both hepatocytes and HMBs.

On the other hand, vitrification caused rupture of microbeads structure in all preparations and fine cracks in intact microbeads. Therefore, vitrification cannot be used for hepatocyte cryopreservation.

Previously published work showed that apoptosis could occur in hepatocytes post cryopreservation, hence in this study apoptosis was assessed in cultured rat hepatocytes (24h post-thawing) using relative-quantification real-time PCR and gene expression assays for Bcl2, Bax, Casp3, Casp9, and GAPDH (housekeeping) genes. The results showed that the cryopreservation solution UW supplemented with 60µM Z-VAD, 5% glucose, and 10% DMSO (cond1) gives better results suggesting that cells had a "better" protection against apoptosis compared to the other four cryopreservation conditions. However, it would be ideal to carry out further studies to investigate the use of other cryoprotectant agents that may have enhanced anti-apoptotic properties.

In conclusion, an optimised protocol for cryopreservation of hepatocyte microbeads was achievable, were readymade cryopreservation solutions Cell Banker1, and Cryo-JIN resulted in improved hepatocyte functions and activity compared to UW control. Moreover, UW solution supplemented with 10% DMSO, 0.2M Trehalose, 60µM Z-VAD enhance hepatocyte, and hepatocyte microbeads cryopreservation outcome compared to control. This modified protocol using one of these solutions could be used in the future for cryopreservation of clinical grade hepatocyte microbeads.

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List of Abbreviations

%	Percent
℃	Degree Celsius
ALF	Acute liver failure
ANOVA	two-way analysis of variance
BAL	Bio Artificial liver
BSA	Bovine serum albumin
Ca+2	Calcium ion
cDNA	Complementary DNA
CPA	Cryoprotectant
CRF	Controlled-rate freezer
CYP450	Cytochrome P450
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene glycol triacetate acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimal essential medium
ETK	ET-Kyoto solution
EG	Ethylene glycol
EROD	Ethoxy Resorufin-O-Demethylase
FCS	Fetal Calf Serum
FDA	fluorescein diacetate
g	gram
GMP	Good manufacturing practice
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HMBs	Hepatocyte Microbeads
HES	Hydroxyethyl Starch
Hz	Hertz
ITS	insulin-transferrin-selenium
kDa	Kilo Dalton
kV	kilo voltage
LN	Liquid Nitrogen
LDH	lactate dehydrogenase
mg	Milligram
min	Minute
mM	Millimole
µM	Micromole
MTT	3-(4.5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide
MW	Molecular weight

OD	Optical density
OLT	Orthotopic liver transplant
PBS	Phosphate buffered saline
PI	Propidium iodide
PLL	Poly-L-Lysine
PEG	Polyethylene glycol
PVP	Polyvinyl pyrrolidone
pH	Hydrogen number concentration
sec	Second
RNA	Ribonucleic Acid
SRB	Sulphorhodamine B
SD	standard deviation
v/v	volume to volume
V	voltage
UW	University of Wisconsin solution
WEM	William's E Medium
Z-VAD	Benzyloxycarbonyl-Val-Ala-DL-Asp-fluromethylketone

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Chapter 1

Introduction

1.1. Acute Liver Failure

Acute liver failure (ALF) is a disease that carries a high mortality rate among the world (Blackmore & Bernal, 2015 & Thanapirom et al., 2019), ALF sometimes called "fulminant hepatic failure" or "acute hepatic necrosis" (Akamatsu, Sugawara, & Kokudo, 2013). ALF could happen due to a sudden cause and leads to several health complication such as cerebral edema, coagulopathy, renal failure, metabolic disturbance, hemodynamic instability, and susceptibility to infection (Mazumder, Begum, & Karim, 2017), Especially for young infants, who could develop sever complication in short period, and leads to death mainly due to shortage availability of suitable donor organs (Cardoso et al., 2018).

Children are susceptible to ALF, however the causes of ALF in children differ from those in adults and vary according on the child's age. There are many factors affecting the aetiology of ALF with children such as age, inadequate defensive resources, immaturity of organs or systems (in new-borns and babies), or problems unique to the childhood era that can be exceedingly dangerous (metabolic disorders). Infectious reasons and metabolic problems are more frequent in new-borns and babies, toxicants, autoimmune diseases, and Wilson's disease (WD) are all involved in older children and teens.(Grama et al., 2020) furthermore; tyrosinemia, hereditary fructose intolerance, glycosylation defects, urea cycle defects are other causes of ALF (Mitry et al., 2003).

Management of ALF is possible with optimal medical therapy, especially when identifying the causes of ALF, this will definitely plays a vital role in ALF management (Grama et al., 2020).However some cases could require orthotopic liver transplantation (OLT)

as a radical treatment for ALF(Akamatsu et al., 2013). In cases of shortage of matching donors, OLT could not be the available solution, therefor hepatocyte transplant could be the best available alternative. Kings College hospital; hepatocyte transplant team have developed hepatocyte transplantation treatment for children with ALF, by using cryopreserved isolated hepatocytes from donor livers (Iansante et al., 2018). Hepatocytes are demonstrated to the patient liver via the hepatic portal vain and native liver remains in place as a backup in case of cell graft failure and allowing potential regeneration in patients with acute liver failure (ALF), as well as representing a potential target for future gene therapy in patients with liver-based metabolic disorders; this procedure is very safe and simple, which requires a fraction of the isolated hepatocytes from a donor organ, therefore, multiple procedures can be performed for the same patient (Dhawan et al., 2010).

In most cases immediate liver transplantation is recommended; hepatocyte transplantation is considered as a potential alternative treatment. Even though current medical and surgical therapies are available for early stages of liver diseases, significant developments are needed for alternative treatments to be successful. Cell-based therapies are being developed as promising tools, alternative to orthotopic liver transplantation (OLT). Human hepatocytes which isolated from unused or rejected livers can be cryopreserved and banked, for emergency clinical use. In fact, human hepatocyte transplantation is emerging as an alternative to liver transplantation for the treatment of liver-based metabolic diseases, and acute liver failure (Dhawan et al., 2010 & Cardoso et al., 2017).

Furthermore, King's College hospital have successfully used alginate-encapsulated hepatocytes (hepatocyte microbeads; HMBs) transplantation for treatment of children with ALF as an alternative method to OLT. The results showed safety and feasibility of the technique with very encouraging outcome, where some patients recovered without the need for liver transplant (Iansante et al., 2017). The main aim for using HMBs is to buy the patient time

until a suitable organ is available for transplantation. However, as the liver has a regenerative potential, the transplanted HMBs may support the failing liver resulting is sufficient restoration of the missing liver functions and recovery of the patient without the need for liver transplantation.

The encapsulated cells are delivered into the peritoneal cavity, in a simple and safe procedure, and without the need for immunosuppression drugs. Currently HMBs are freshly prepared before intraperitoneal transplantation. It would be ideal if cryopreserved HMBs readily available for emergency cases., Encapsulation protects the hepatocytes from the host immune attack and at the same time allows oxygen and nutrients to diffuse to cells as well as waste products such as urea and vital products such as coagulation factor VII and albumin diffuse out of HMBs.

Cryopreservation can cause damage to HMBs including ice formation during freezing, and post-thawing. However, previously published pre-clinical studies showed that alginate encapsulation may protect hepatocytes from freezing and post-thawing damages. Jitraruch et al., (2014) established an optimised protocol for the production of HMBs. Therefore, optimising a protocol for the cryopreservation and banking of clinical grade HMBs is needed (Iansante et al., 2017).

Different types of cryopreservation solutions and cytoprotectants which potentially can be used for clinical application could be used for optimizing microbeads cryopreservation protocol. The initial study on cryopreservation of microbeads findings is very promising (Jitraruch et al., 2017). Success of this proposed research project will make banked cryopreserved hepatocyte microbeads available for emergency cases such as Acute Liver Failure (ALF).

1.2. Cryopreservation

Cryopreservation is the process of freezing and storing biological samples at cryogenic temperatures (Karlsson & Toner, 1996). The biological samples could be cells, tissues, or encapsulated cells (Wu et al., 2007). Hepatocyte cryopreservation is important for the purpose of hepatocyte transplantation as well as the bioartificial liver support system (BAL). However, cryopreservation induced damaged due to the ice formation is the main obstacle facing the scientists (Wu et al., 2007). Optimized cryopreservation protocols are needed to maintain hepatocyte viability and activity post thawing as possible as it can be. The addition of cryoprotectants, anti-freezing agents, ice nucleating agents, pan-caspase inhibitors or ice blockers could enhance the cryopreservation mechanism , enhance the retrieval of viable cells and prevent freezing induced damage during cryopreservation process (Jitraruch et al., 2017).

There are two main methods for cryopreservation; the ***slow freezing*** using the controlled-rate freezer (CRF), and the rapid freezing method, ***vitrification***, where the samples are immersed into the liquid nitrogen immediately. In slow freezing method; temperature decreases gradually to reach cryogenic temperature, this can be achieved by using CRF or cell cooler (known as "Mr Frosty") (Royere et al., 1996).

1.2.1. Slow freezing (Controlled rate freezing)

Controller rate freezer or controlled rate cooling is the most commonly used device for the slow cryopreservation method, the device is supported by controlled injection of liquid nitrogen to cool the samples in gradual rate with the presence of the cryoprotectants (Baust et al., 2016). Cryopreservation using the controlled rate freezer is believed to minimize the ice formation during the cryopreservation, by adjusting the declining of the temperature and the loading of the cryoprotectants in low concentration to avoid the ice formation (Buhl et al., 2012).

1.2.2. Vitrification

Vitrification is processes where liquids drives from the liquid phase immediately to the solid phase, glass forming, without giving the chance for the ice formation (Wang et al., 2012). Vitrification can be an alternative method for the controlled rate freezer for the purpose of long-term cryopreservation of cells or tissues. In this method high cryoprotectant concentration are applied to prevent the ice formation 4-8 M (Heo et al., 2015). The samples are immediately impeded in to the liquid nitrogen to form the glass solid phase and avoided ice formation (Heo et al., 2015). Wu et al. (2007) proved that vitrification can be used for the cryopreservation of encapsulated hepatocyte.

Vitrification improves cryopreservation by eliminating the mechanical injury from ice formation (Jang et al., 2017). Vitrification is used to cryopreserve many types of cells and tissues including cells, tissues, organs, Oocytes, sperms and some organisms (Gandolfi et al., 2006). To achieve good vitrification results, its preferable to load the samples before coaling into the cryopreservation solutions with the cryoprotectants. This will avoid ice formation during the entire freezing process (Wowk, 2007). During freezing, salts and cryoprotectants reduces ice formation within the cell by osmosis, as the cooling is continued, ice separates and salt in the remaining liquid, is more concentrated therefore lower chance of ice formation exist (Chian & Quinn, 2010). A better understanding of vitrification will allow scientist to improve present results, and develop more vitrification solutions in the future (Adams et al., 2015).

Karlsson and Toner (1996) reported that rapid cooling during vitrification does not allow cell dehydration leading to amidite glass formation. However, cooling and warming rates plays an important role for cell retrieval post cryopreservation as stated by Rival theories of freezing injury, where he investigated that cryoprotectant could prevent ice formation in low temperature and should not be added with lethal concentration (Pegg, 2007).

1.3. Hepatocyte cryopreservation

Limited availability for fresh hepatocytes called for the need of developing hepatocyte cryopreservation protocols, hepatocyte cryopreservation is important for many research studies such as drug metabolisms, cytotoxicity testing, and clinical application of hepatocellular transplantation and many other liver diseases. optimization and validation of cryopreservation techniques is key to restore the banked hepatocyte for future uses.

Hepatocyte cryopreservation gave the chance to store the hepatocyte for further research or clinical uses, many drugs approval have been done through the uses of cryopreserved hepatocyte. hepatocyte cryopreservation enabled more research on liver models and diseases as well as drug efficacy testing. On the other hand, cryopreservation could lead to hepatocyte damage and in turn might reduce the viability and hepatocyte-specific functions, therefore, the addition of cryoprotectant or anti freezing agent could reduce the cryodamage and maintain the viability of hepatocyte upon thawing.

Hepatocytes are very sensitive and fragile and affected by Freeze-Thaw process, and they do not proliferate in culture, they only can be cultured and maintained with the hepatic specific function (Grondin et al., 2009).

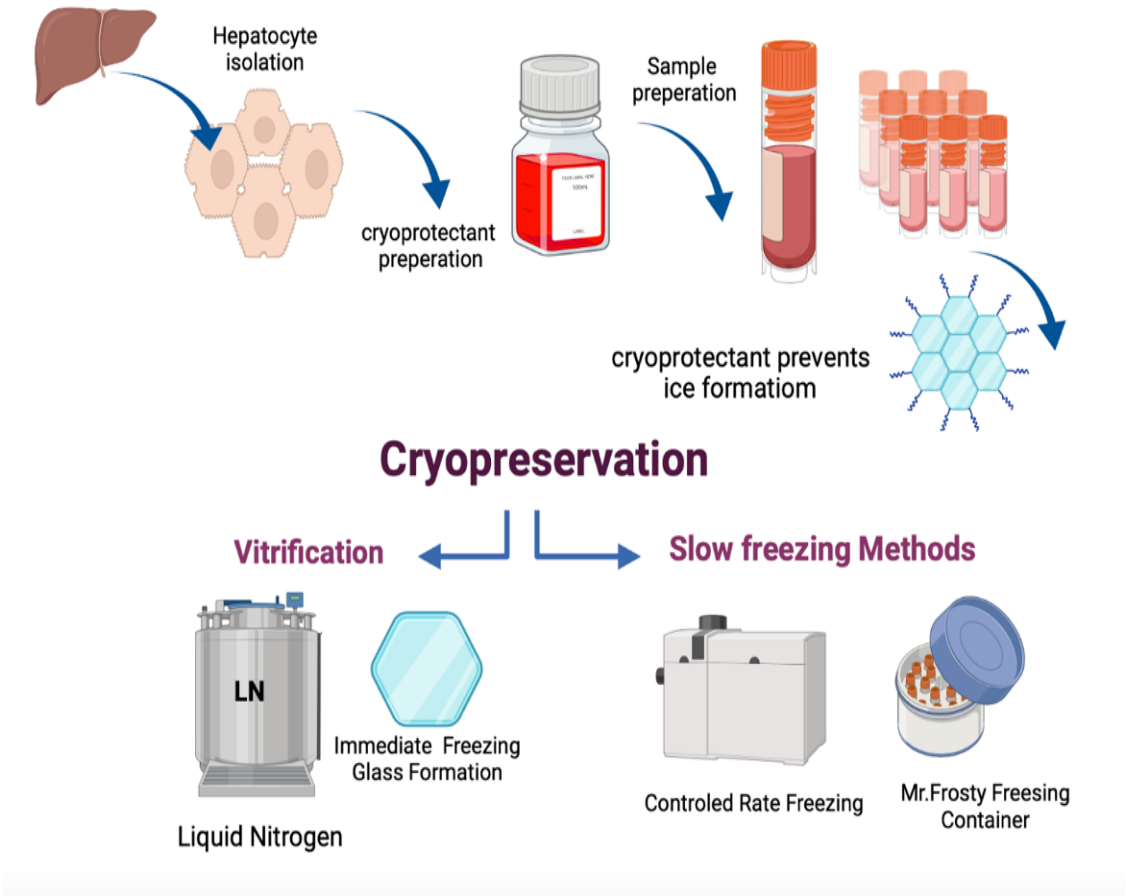


Figure 1.1: Main Types of cryopreservation, Vitrification, and the Slow Freezing

[Illustration created using BioRender platform, BioRender.com]

1.3.1. Cryopreservation induced damage

Ice formation at low temperatures is one of the main causes of cell freezing induced damage, leading to cell death, and in order to reduce ice formation during cryopreservation process, cryoprotectants have been introduced. CPAs can be defined as chemicals or compound that dissolve in water and helps lowering the melting point of the water (Devismita & Kumar, 2015). Different studies showed that the addition of the CPAs could enhance the cryopreservation and result in better cell viability and function post thawing (Terry et al., 2006).

1.4. Cryoprotectants (CPAs)

Cryoprotectants (CPAs) are substances added to the cryopreservation solutions in order to reduce cells cryodamage during the freezing process (Bhattacharya & Prajapati, 2016a), CPAs can be classified as (Intracellular) Permeable cryoprotective agents (PCPAs) or (Extracellular) non-permeable cryoprotective agents (non-PCPAs), based on their ability to penetrate the cell membrane (Pan et al., 2017). Cryoprotectants were first introduced by Polge et al. (1949), and in their experiments they have used glycerol to cryopreserve avian sperms, and they discovered that Glycerol could be used as a cryoprotectant. later in 1959, Lovelock and Bishop reported the use of DMSO as a cryoprotectant and its advantages during cryopreservation. These two studies identified the benefits of adding cryoprotectant(s) to enhance the cryopreservation outcome (Baust et al., 2009).

Cryoprotectants can be classified as Penetrating, and Non-penetrating Cryoprotectants based on their ability to diffuse through the cell membrane (Wang et al., 2012). Penetrating cryoprotectants are small molecules which are able to cross cell membranes and create hydrogen bound with water molecules leading to lowering the freezing point (Yong et al., 2020). Moreover, penetrating cryoprotectants function in two different ways: a) reducing the osmotic stress during freezing, and b) prevent ice formation (Yoshida et al., 2020). On the other hand, non-penetrating cryoprotectants are large compounds, such as polymers, sugars, and sugar alcohols. They inhabit ice formation and maintain cell integrity; examples of commonly used of such cryoprotectants are Polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Non-penetrating cryoprotectants are usually less toxic than penetrating cryoprotectants at the same concentration (Best, 2015).

Cryoprotectants also can be classified based on their chemical characteristics to; ***Alcohols and derivatives***, ***Sugars and sugar alcohols, Polymers, Sulfoxides, and Amides and Amines.*** Each category has its specific function(s) and reactions during cryopreservation. ***Alcohol and Derivatives*** such as; ***Methanol, Ethanol, Glycerol, Propylene glycol and Ethylene glycol***. For example, ***Glycerol;*** is a sugar alcohol, very viscous liquid, also known as glycerine. Glycerol is cosolvent which can form hydrogen bonds with water molecules. In 1949, Polge and colleagues reported the discovery of glycerols as cryoprotectant agent to preserve avian spermatozoa (Jang et al., 2017). Glycerol has been the primary cryoprotectant for freezing human semen since the first successful procedures were reported in 1953 (Hammitt et al., 1988).

Ethylene glycol (EG), plays a key role in changing the water hydrogen bounding in the solution. EG freezing point is -12°C, however when it is mixed with water (6:4; v/v) the freezing point drops to -45°C. Therefor EG is considered as one of the most effective CPAs (Bhattacharya & Prajapati, 2016a).

Sugars and sugar alcohols such as; Glucose, Galactose, Lactose, *Trehalose, Raffinose, Mannitol, and Sorbitol* are all used as cryoprotectants. Disaccharides such as ***Sucrose*** and ***Trehalose*** have been widely used as natural cryoprotectants. Both sucrose and trehalose, are isomers of a non-reducing disaccharide (Rodrigues et al., 2008). During cryopreservation, the two can balance extra- and intra-cellular osmotic pressures. Studies proven that sucrose and trehalose were the most effective cryoprotectants among all the di-saccharides (Wang *et al.* 2014; Kim *et al.* 2016).

Sucrose is a naturally occurring disaccharide consist of glucose and fructose sugars, sucrose in low temperature (−45°C) provides required nutrients to preserved cells. A combination of DMSO and sucrose maintains good cytoprotective properties (Bhattacharya & Prajapati, 2016b). Another study done by Nguyen and Truong (2014), where they attempted

to protect catfish embryos from cryo-damage. They found that combining 0.5M sucrose with 1 M propylene glycol protected the catfish embryos from cryodamage between (0°C and -20°C). Furthermore, a study by Pan et al. (2017) tested the efficiency of the sucrose during cryopreservation of pig spermatogonial stem cells (pSSCs), using different concentrations of sucrose, the study showed that sucrose promoted pSSCs survival after freezing.

Trehalose is disaccharide, consists of two glucose molecules joined by a,a-1,1-glycosidic linkage, also known as mycoses or mushroom sugar. It naturally is found in high concentrations, as many organisms including microorganisms, plants, and invertebrates can survive in dehydrated environment up to 99% water loss, this phenomena is so called anhydrobiosis (Rodrigues et al., 2008), and therefore are able to survive at cryogenic temperatures (Takahashi et al., 2006). During cryopreservation trehalose interacts with the phospholipids in the plasma membrane of the cells, forming a glassy coat around the cells protecting them from the extra cellular ice damage (Yong et al., 2020).

Some studies reported that trehalose stabilises the cell membrane and protein during freezing, while others have found that it is more effective when used in combination with other CPs (Beattie et al., 1997). Some studies have also showed that using 10% DMSO and 10% FCS, diluted in culture medium in addition to trehalose enhanced the cryopreservation outcome (Perkins, 2007). Moreover, combination of catalase and trehalose in freezing medium resulted in better cryopreservation (Rodrigues et al., 2008). For example earlier studies indicated that combination of trehalose and DMSO (permeable CAP) improved cell viability post-thawing (Yoshida et al., 2020). When trehalose was combined with DMSO for human hepatocytes cryopreservation, a significant increase in total protein level and secretion of albumin was observed post-thawing (Stéphenne et al., 2010). Studies on mouse and bovine spermatogonial

stem cells reported that sucrose and trehalose were the most effective cryoprotectants among all the disaccharides tested (Len, Koh, & Tan, 2019).

In a study done by Yoshida et al. (2020), they showed that low concentration of trehalose (2.6 mM) improved hepatocyte cryopreservation dramatically and improved cell viability and function post-thawing (Yoshida et al., 2020).

Katenz et al. (2007) tested different concentration of trehalose for human hepatocyte cryopreservation, and they showed that 0.2 M trehalose resulted in best cryopreservation outcome compared to other concentrations. A study conducted by Illouz et al. (2008) to test the cryopreservation effectiveness of ET-Kyoto solution (ETK), which is a trehalose-containing organ preservation solution, showed that ETK gave satisfactory results for the cryopreservation of primary human hepatocytes. This shows that trehalose is beneficial as cryoprotectant for hepatocyte cryopreservation (Illouz et al., 2008).

Another study reported by Cardoso et al. (2017) investigated the effectiveness of trehalose (0.2 M) for hepatocyte cryopreservation, showed that 7 days post-thawing, hepatocytes had good viability and activity. compared to other disaccharides tested (Cardoso et al., 2017).

Polymers, Some polymers can be use as CPs such as Polyethylene glycol (PEG), Polyvinyl pyrrolidone (PVP), Dextrans, Ficoll, Hydroxyethyl Starch (HES), Serum proteins, milk proteins and Peptones (Hubálek, 2003).

Sulfoxides and Amides, are another class of CPAs and have been frequently used for cryopreservation of different cell types, examples of such commonly used CPAs are Dimethyl sulfoxide (DMSO), Acetamide, Formamide, and Dimethyl acetamide. DMSO is a colourless solution and organosulfur derivative with molecular formula $(CH_3)_2SO$. In 1866, it was

synthesized by the Russian scientist Alexander Zaytsev. DMSO can dissolve both polar and non-polar compounds, and this property makes it a very good cryoprotectant (Bhattacharya & Prajapati, 2016a). In 1959, Lovelock and Bishop reported the advantages of using DMSO as a cryoprotectant during cryopreservation of different types of cells compared to glycerol. One of the main characteristics of the DMSO, is it can enter the cells and reduce electrolyte concentration in the un-frozen solution, therefore reducing ice formation (Baust et al., 2009). In addition to the amines, Proline, Glutamine and Betaine are also considered as good CPAs (Elliott et al., 2017).

Furthermore, some other compounds could be used as cryoprotectants such as crude wheat extract, in a study done by Grondin et al. (2009), they tested it for rat hepatocyte cryopreservation. The study showed that crude wheat extract could provide a promising alternative to DMSO. The advantage of using wheat proteins as cryoprotectant is that its non-toxic, natural products and do not contain any animal protein or serum, and it can be easily extracted and prepared (Grondin et al., 2009).

Table 1.1: Types of Cryoprotectants (Elliott et al., 2017)

Alcohols and derivatives	***Sugars and sugar alcohols***	***Polymers***	***Sulfoxides and Amides***	***Amin's***
– Methanol – Ethanol – Glycerol – Propylene glycol – Ethylene glycol	– Glucose – Galactose – Lactose – Trehalose – Raffinose – Mannitol – Sorbitol	– Polyethylene glycol (PEG) – Polyvinyl pyrrolidone (PVP) – Dextran's – Ficoll – Hydroxyethyl Starch (HES) – Serum proteins – Milk proteins – Peptones	– Dimethyl sulfoxide – Acetamide – Formamide – Dimethyl acetamide.	– Proline – Glutamine – Betaine

1.4.1. Carrier solution for cryopreservation

Carrier solution works as buffering medium during cryopreservation, the purpose of this solution is to preserve cell and maintain its integrity during the freezing process (Fahy et al., 2004). Carrier solution mainly consist of buffers, osmogenes, nutritional elements ice-blockers, and anti-apoptotic compound (Bhattacharya & Prajapati, 2016b). The main purpose of having this solution in cryopreservation prosses is to maintain the osmolarity, cell shape, and medium pH (Wowk, 2007). Adding cryoprotectants and ice blockers as well as anti-apoptotic agent(s) to carrier solution could enhance the cryopreservation outcome. A good example of carrier solution is ***LM5*** which is used in ***M22*** cryoprotectant solution, and it contains 1 mM $CaCl_2$ and 2 mM $MgCl_2$ and many other components listed in Table (2), (Fahy et al., 2004). Carrier solution concentration is always constant, while cryoprotectant concentration can be changed depending on the cell type and the cryopreservation protocol used (Bhattacharya & Prajapati, 2016b).

Table 1.2: Component of M22 Solution; (Fahy et al., 2004)

Components	Concentration
Dimethyl Sulfoxide DMSO	2.855M (22.305% w/v)
Formamide	2.855M (12.858% w/v)
Ethylene Glycol	2.713M (16.837% w/v)
N-Methylformamide	0.508M (3% w/v)
3-methoxy,1,2-propanediol	0.377M (4% w/v)
PVP K12	2.8% w/v (~0.0056 M)
PVA	1% w/v (~0.005 M)
PGL	2% w/v (~0.0267 M)
5X LM5	20ml/dl
PH	8.0
Melting point	-54.9°C

1.5. Ice blockers

Ice blockers are the substance which prevents ice growing during freezing, at low temperature ice blockers binds with ice inculcators and cleave ice formation. The most commonly used ice blockers are polyvinyl alcohol, polyglycerol, called as X-1000 and Z-1000 (Bhattacharya & Prajapati, 2016b).

1.6. Apoptotic inhibitors

Recent studies showed that apoptosis is part of cryopreservation induced cell injury, therefore understanding the apoptosis pathway during cryopreservation could enhance the cryopreservation outcomes (Martin et al., 2004). Apoptotic activation in response to low temperature exposure have been documented in a variety of cell cryopreservation processes including renal cells, fibroblasts, hepatocytes, peripheral blood mononuclear cells, cord blood, spermatozoa, oocytes, ovarian-tissue, vascular tissue and other type of cells (Baust et al., 2009; Bhattacharya & Prajapati, 2016b).

Apoptosis pathway during cryopreservation could occur due to the activation of caspase-8 through the extrinsic pathway or the intrinsic pathway through caspase-9. Therefore, using apoptotic inhibitors could enhance the cryopreservation process (Xu et al., 2010; Len et al., 2019). Two apoptosis inhibitors, Benzyloxycarbonyl-Val-Ala-DL-Asp-fluromethylketone (Z-VAD), and Trans-f-[(1R)-aminoethyl]-N-4-pyridinyl cyclohex anecar–boxamide dihydrochloride (Y-27632) have been used as antiapoptotic agent(s) during spermatogonial stem cell cryopreservation, both showed improvement of cryopreservation outcome (Ha et al., 2016).

Table 1.3: Mode of Action of Antioxidants; (Reed, 2014)	
Antioxidants	**Mode of Action**
Ascorbic acid (Vitamin C)	Quenches free radicals
Catalase	Decomposes hydrogen peroxide
Glutathione	Oxidized by ROS
Glycerol, sucrose and DMSO	Free radical scavengers
Glycine betaine	Activates ROS-scavenging systems, protects plasma membrane proteins
Lipoic acid	Free radical scavenger or signaling molecule
Superoxide dismutase (SOD)	Catalyzes superoxide breakdown to hydrogen peroxide and water
Tocopherol (Vitamin E)	Main membrane antioxidant

1.7. Antioxidants

Many antioxidants such; 2,4-dinitrophenol (DNP), ascorbic acid (Vitamin C), antifreeze proteins (AFP), glutathione, hypotaurine, glutathione peroxidase, Coenzyme Q, egg yolk, superoxide dismutase (SOD), tocopherol (Vitamin E), and catalase have been tested to enhance the cryopreservation outcomes (Reed, 2014). Hypotaurine is also considered as good antioxidant, and in a study by Ha et al. (2016), it was shown that hypotaurine is effective in cryopreservation of mice spermatogonial stem cell (Ha et al., 2016). **Table (3)** list the mode(s) of action of each antioxidant.

1.8. Cryoprotectants toxicity

Cryoprotectants plays an important role in reducing cell damage at low temperature where ice formation can happen and causes cell fraction and nucleation, however selection of cryoprotectants should be done very carefully, and added to the solution in molar concentration, because excess amount of CPAs could be lethal to cells, consequently could

cause apoptosis which is unfavourable for cryopreservation goals (Bhattacharya & Prajapati, 2016b). When using the slow freezing methods, low concentration of CPAs are needed less than 2 M, while high concentrations are required for the vitrification method (4-8 M) (Zheng et al., 2018).

1.9. Warming

During thawing or devitrification, it is more likely that ice-formation occurs, where a process of recrystallization could happen. Therefore, to minimize damage due to devitrification, and recrystallization, rapid warming is recommended especially for the vitrified samples (Karlsson & Toner, 1996).

Devitrification and recrystallization are more likely to exist in rapidly cooled cells, because the cytoplasmic supercooling which leads to intracellular ice nucleation (Karlsson & Toner, 1996). However, in some cases rapid warming rates can yield lower post- thaw survivals than slow rates, especially for cells that have been frozen using slow rate freezing. Until now, the exact mechanism by which warming process could affect cell survival is not fully understood, however rapid warming rates appear to be beneficial for rapidly cooled samples, while slow warming rates may be preferable for slowly cooled samples. On the other hand the concentration of some cryoprotectants could be lethal especially during the warming process, as they are usually nontoxic at lower temperature(s), for example DMSO is toxic at temperatures above than 25°C depending on extent of exposure time (Karlsson & Toner, 1996).

1.10. Cell Encapsulation

Encapsulation is a process where cells are coated with permeable membrane to protect the cells from the host immune system (Nicodemus & Bryant, 2008). Therefore, the main aim of the encapsulation processes is to protect the transplanted cells from the immune attack of the body receiving the cells. However, there are several characteristic and consideration should be undertaken when choosing the encapsulation material to provide similar in vivo conditions to the encapsulated cells (Hunt & Grover, 2010). The challenge was to find a way to encapsulate the cells with membrane that have enough wall thickness, with good and enough permeability that permit the distribution of nutrients to the cells as well as secreting the waste out and have good biocompatibility. This means that the membrane should be very selective in its permeability and must block the immune response from attacking the cells (Nicodemus & Bryant, 2008).

1.11. Bio-compatibility of the encapsulation material

The material chosen for the encapsulation should have the ability to save the cells from the immune response of the host and not cause any inflammatory response to the host, never the less the life time of the encapsulation material should be considered, as biodegradation of the polymerised structure around the cells could lead to immune response, and other complications within the host body (J. Z. Wang, Ding, Zhang, & Ye, 2017).

Therefore, semipermeable are suitable for cell encapsulation, there are different material which can be used as semipermeable membranes for cells encapsulation such as: ***(i) hydrogels***, ***(ii) thermoplastic polymers***, and ***(iii) non-polymeric materials***. Hydrogels are the mostly commonly used encapsulation material, due to its favourable characteristics for cell encapsulation. It consist of hydrophilic polymers which are able to absorb water and dose not

dissolve, as well as they have less inflammatory response comparing to the other types (Caliari & Burdick, 2016). Hydrogels classified into three groups depending on their composition, ***Naturally deriver*** such as: collagen, fibrin and alginate, ***Synthetic Materials Hydrogels*** like: polyacrylamide and polyethylene glycol, and ***Hybrid Materials Hydrogels*** that combine elements of synthetic and natural polymers like: hyaluronic acid and polypeptides (Caliari & Burdick, 2016). On the other hand, thermoplastic polymers are affected by the temperature, their physical properties can be change by the heat. The common thermoplastic used for encapsulation are: acrylonitrile vinyl chloride copolymer (PAN-PVC), but they have low water permeability (Caliari & Burdick, 2016).

Alginates is the most commonly used material for the encapsulation of cells, due to its good permeability, ionotropic properties as well as biocompatibility (Leong et al., 2016). Alginate is considered as a natural polymer extracted from the bacteria and brown seaweeds (K. Y. Lee & Mooney, 2012). It consists of bi-polymers of 1-4 linked β-D-mannuronic acid (M Blocks) and α-L-glucuronic acid (G blocks) in alternating blocks (Daemi & Barikani, 2012), (Figure 1.3). Polymerization takes place in the presence of cations such as calcium or barium or aluminium crosslink with the carboxyl group (Kim et al., 1998).

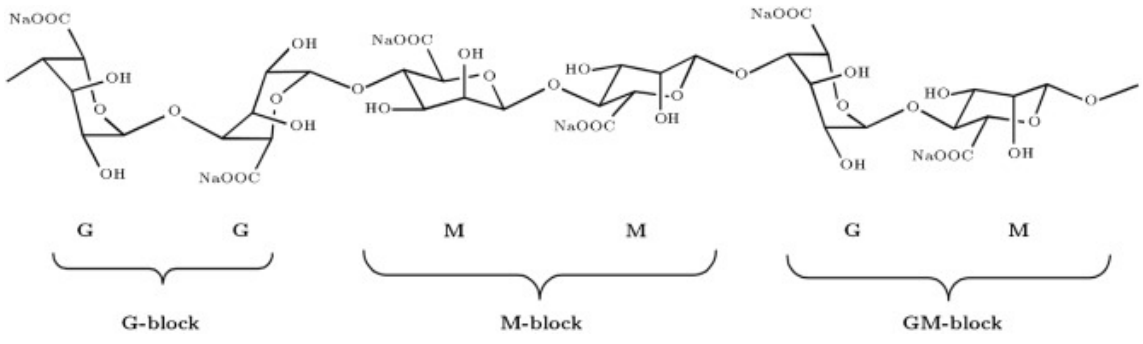


Figure 1.2: Alginate polymer showing the arrangement of (M-Blocks) and (G-Blocks).

1.12. Cell Encapsulation Techniques

Cell encapsulation have wide range of application in the clinical field, especially that the encapsulation will provide immuno-isolation of the cells, and therefore cells could be transplanted without the need for immunosuppression (Young et al., 2012). There are different cell encapsulation methods developed that mainly include: extrusion, lithography, emulsion, microfluidics, bioprinting, and superhydrophobic surface(J. Z. Wang et al., 2017). Extrusion is the most commonly used technique, depending on gravitational dripping of liquid solution of the cells and the encapsulation material mixed then passed through a needle and extruded with the gravitational dripping in polymerization solution (example: $CaCl_2$) where spherical microbeads will form (Nedovic et al., 2011). Usually vibrational encapsulators are used for this technique with controlled vibration through the nozzle and electro spraying by applying electrical potential at the tip of the nozzle or what is known by high voltage electrostatic pulse system (Al-Rammah, 2014). In this technique, small droplets production are ensured by coaxial air flow, where air jet around the nozzle with the vibration leads to the brakeage of the liquid into small droplets. These small droplets will polymerize into small spherical microbeads in the polymerization solution (Al-Rammah, 2014). Producing uniform size and shape of microbeads depends on three main parameters: voltage (V), the impulse frequency (f), and time (T); by controlling these parameters will enable to form microbeads with a size of 0.2-3.0 mm (Al-Rammah, 2014).

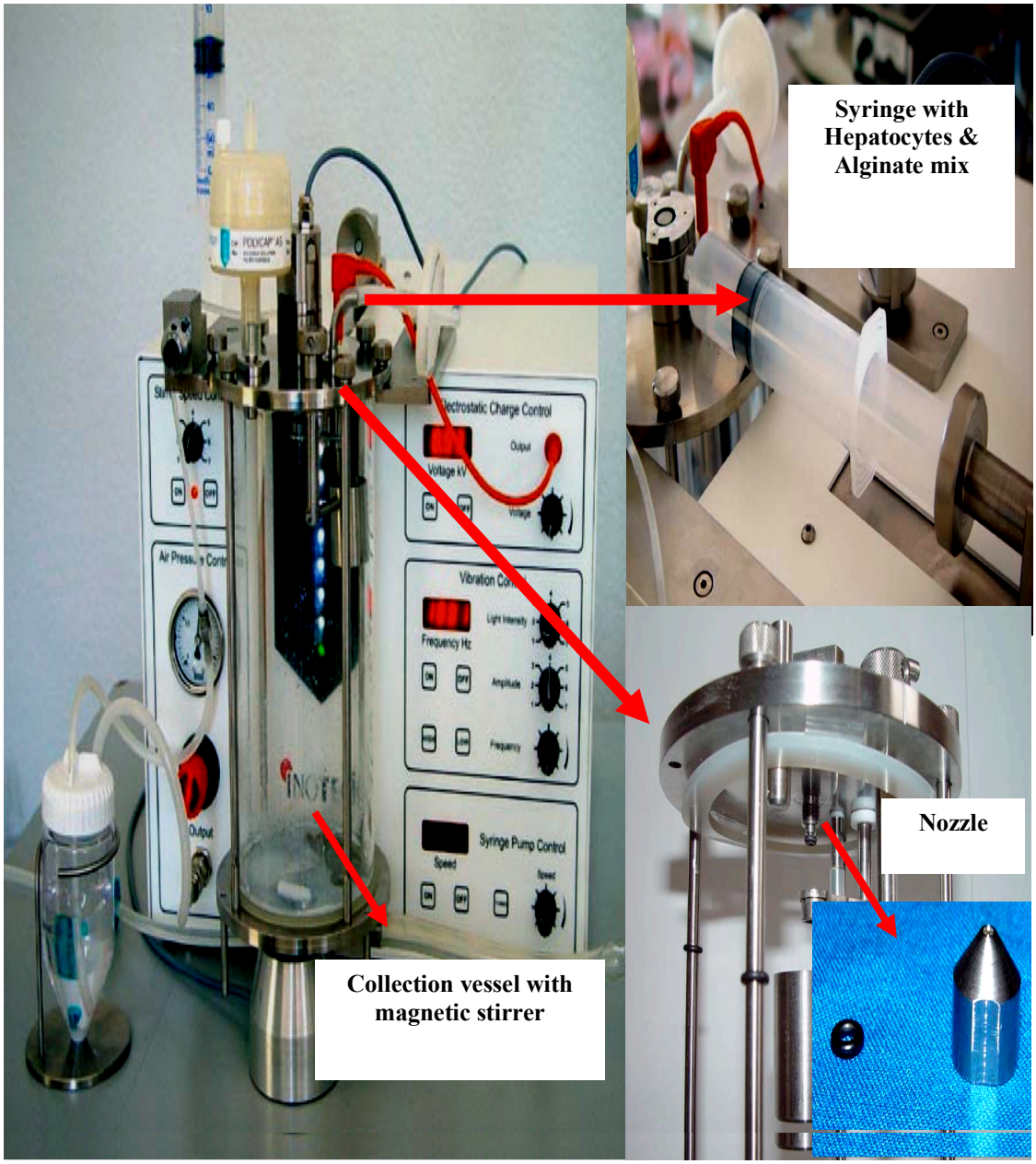


Figure 1.3: Encapsulator used in this study; Büchi model: Inotech R-50.

1.13. Hepatocyte Encapsulation

The first cell encapsulation was introduced in 1933 by Bisceglie, where tumour cells were encapsulated with polymer membrane and transplanted in the abdominal cavity of pigs. It was shown that the cells were able to survive for long time, and function very well without being attacked by the host immune system (Orive et al., 2004). After thirty years, Chang came with the "artificial cell" which are encapsulated cells to work as immuno-protection for the transplanted cell (Orive et al., 2004). In 1970 the practice of cell encapsulation was successfully approached with pancreatic islet cells in diabetic animal models, followed by the development of the microencapsulator, therefore cell encapsulation had been used in a wide range of therapeutic applications worldwide **(Table 1.1)**.

King's College Hospital pioneered transplantation of human encapsulated hepatocyte in children with acute liver failure, where they used alginate as encapsulation polymer. The transplantation was very successful and the encapsulated hepatocytes survived survive for 6 months in the peritoneal cavity of the patients without the need of the immunosuppression (Jitraruch et al., 2014).

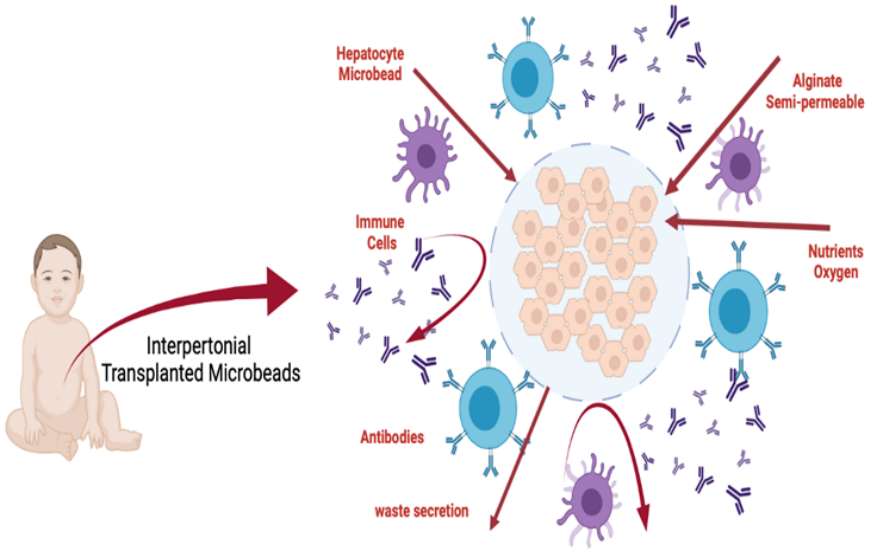


Figure 1.4: Hepatocyte Encapsulation and its Micro-environment within the Body

[Illustration created using BioRender platform, BioRender.com]

Table 1.4: Application of Encapsulated Cells	
Cell Type	**Application**
Fibroblasts	Metabolic deficiencies, neurotrophic factors, epilepsy
Myoblasts	Metabolic deficiencies, neurotrophic factors, cancer
Kidney cells	Haemophilia, neurotrophic factors, anti-angiogenesis
Pancreatic islets	Diabetes
Ovary cells	Fabray disease
Parathyroid cells	Artificial organs
Hepatocytes	liver transplantation
Chondrocytes	Bone and cartilage regeneration
Leydig cells	Hormone replacement
Adrenal chromaffin cells	Parkinson's disease, chronic pain
Stem cells	Bone regeneration
PC12 pheochromocytoma cells	Neurotrophic factors, neurotransmitters
Myeloma cells	Hepatic growth factor
Hybridoma cells	Antibody production
Tumour cells	Cancer vaccine, interleukins
Virus producer cells	Cancer

1.14. Hepatocyte Microbeads Transplantation

Cryopreserved Hepatocyte Microbeads have been tested for it efficiency to cure acute liver failure ALF and different liver diseases. In a study carried out by Baldini et al. (2008) to assess the viability and function of cryopreserved porcine hepatocyte microbeads at -196°C for one month, then transplanted in rat peritoneal cavity without immunosuppression.

The hepatocyte microbeads were then explanted and assessed for function and viability. The results showed that long term cryopreservation of hepatocyte microbeads result in retention of their biological activity (Baldini et al., 2008). Another study by Aoki et al. (2005) at Showa University, Tokyo where human and rat hepatocyte were encapsulated with poly-L-lysine alginate. The hepatocyte microbeads were cryopreserved using DMEM media with 10% FBS and 10% DMSO and frozen immediately in liquid nitrogen. One month later cryopreserved human hepatocyte microbeads were transplanted in rat spleen, without immunosuppression (Aoki et al., 2005). The transplanted cryopreserved HMBs showed good retention in both cell survival and viability at rat spleen (Ogawa et al., 2014).Therefore its believed that cryopreservation of encapsulated hepatocyte will solve the shortage of donor livers and will advance the hepatocyte transplantation. In 2009, at University Hospital Geneva, a study done by Mei et al. (2009) pig cryopreserved encapsulated hepatocytes were transplanted in mouse model of fulminant liver failure, and it was shown that the cells were viable and functioned for two weeks post-transplantation. Nevertheless, a study done by Sgroi et al. (2011) found that intraperitoneal transplantation of encapsulated human hepatocytes in mice with ALF, resulted in a significant survival of the mice and recovery of liver function (Sgroi et al., 2011). As mentioned above, King's College Hospital is the first centre worldwide to transplant hepatocyte microbeads in human. Due to shortage of donor organ, and fresh hepatocytes, and the low viability of cryopreserved hepatocytes post thawing, the microbeads production and bio-banking could overcome these problems.

Table 1.5: Animal Models for Liver Diseases		
Human liver problem	**Animal model**	**Reference**
Crigler–Najjar type 1	Gunn rat	Le Blanc et al., 2003.
Familial hyper-cholesterolemia type 1	Watanabe rabbit	Dunay et al., 2011.
Progressive familial intrahepatic cholestasis	multidrug resistance prote	Azuma et al., 2007.
Wilson's disease	Long–Evans Cinnamon rat	De Vree et al., 2000.
X-linked severe combined immunodeficiency	recombination activation gene 2 mouse	Malhi et al., 2008.

1.15. Hepatocyte Transplantation Pre-Clinical Studies (Animals models)

Pre-clinical hepatocyte transplantation (HT) started in 1970s, and was shown to be successful animal models (Mitry et al., 2011). There are several published studies of hepatocyte transplantation in animal models of liver diseases including the Crigler–Najjar syndrome type I (Gunn rat), hypoalbuminemia using (Nagase albuminemic rats), ornithine transcarbamylase deficiency (Spf-ash mice), and hyperuricosemia in dogs (Le Blanc, Tammik, Rosendahl, Zetterberg, & Ringdén, 2003). These studies showed the possibility of achieving medium to long-term improvements in the liver-specific missing activities. In the case of ALF, rodent models were tested using liver ischemic injury, D-galactosamine, and 90% hepatectomy (Azuma et al., 2007). These studies showed that HT had good improvements in the animal condition and survival.

1.16. Alginate-encapsulated hepatocyte transplantation in ALF

Intraperitoneal transplantation of human microbeads (HMBs) was successful in children with ALF (at King's) and the results were very promising. More patients are being recruited; therefore, production of human hepatocyte microbeads will be in demand.

1.17. Hepatocyte Microbeads cryopreservation

Cryopreservation of microbeads will enable the banking of HMBs for emergency cases. Nevertheless, previous data reported by Jitraruch et al. (2017) at King's College Hospital, and Massie et al. (2011) at UCL Centre of Hepatology showed good results of HMBs cryopreservation using UW solution.

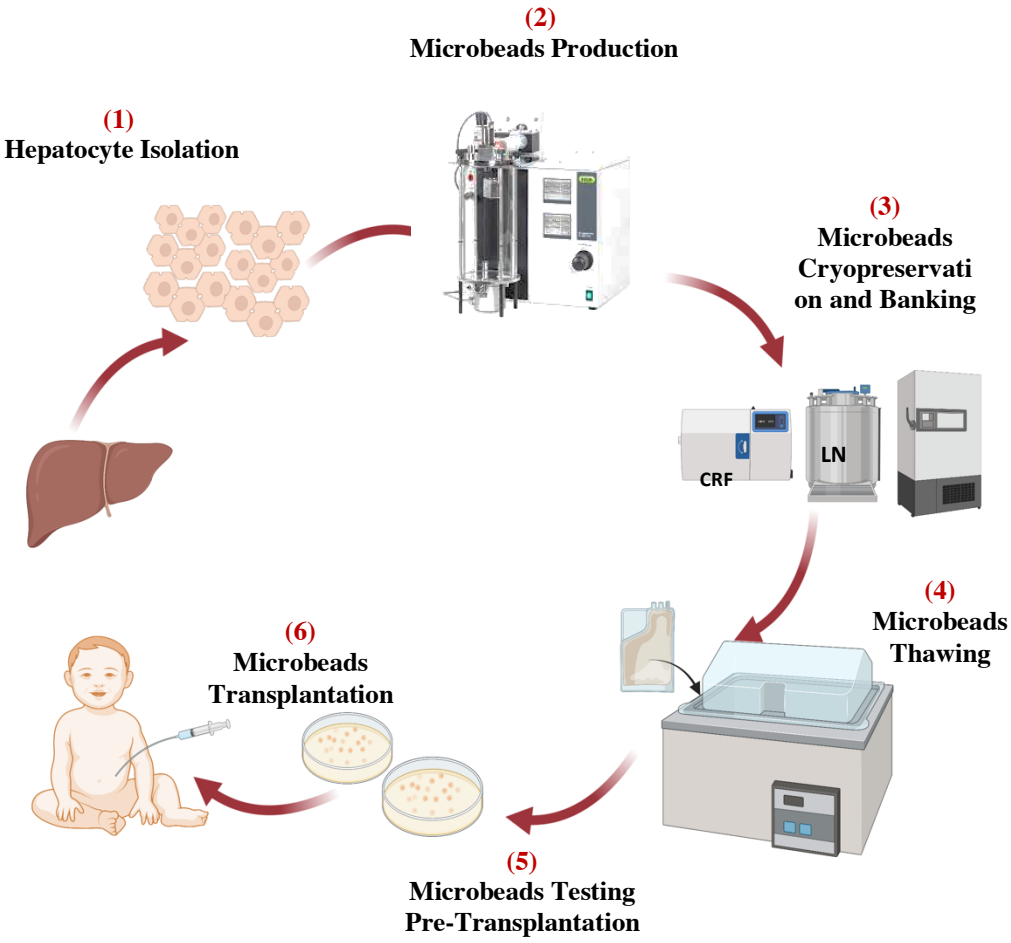


Figure 1.5: A summary of Steps Involved in Microbeads Production and Transplantation: **1)** hepatocyte isolation, **2)** hepatocyte encapsulation (microbeads), **3)** cryopreservation and banking, **4)** microbeads thawing at time of use, **5)** microbeads testing pre-transplantation, and **6)** transplantation.

[Illustration created using BioRender platform, BioRender.com]

1.18. Hypothesis and Aim

The *central hypothesis* of this thesis is that an optimised cryopreservation of human hepatocyte microbeads (HMBs) protocol could be developed and validated for future use for clinical grade HMBs.

The main *aim* of my project is to optimize a protocol for the cryopreservation of the hepatocyte microbeads for the clinical emergency uses. The work will be carried out using different types of cryopreservation solutions supplemented with different types of cryoprotectants, cryopreservation will be conducted using controlled-rate freezing and vitrification technique. The successful modified cryopreservation protocol could set the basis for the cryopreservation of clinical grade hepatocyte microbeads for future use.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Human Hepatocyte isolation buffer solutions and chemicals

- Eagle's Minimum Essential Medium (EMEM) without phenol red or calcium or magnesium (Lonza, Belgium),
- Hank's balanced salt solution (HBSS) without calcium or magnesium, (Lonza, Belgium),
- 1M 4-(2-hydroxyethyl)-1-piperrazineethanesulphonic acid (HEPES; Lonza, Belgium)
- Ethylene glycol tetra acetic acid (EGTA; Sigma-Aldrich Ltd., UK).
- Collagenase P (Roche, UK).

2.1.2. Rat Hepatocyte isolation buffer solutions and chemicals

- Isoflurane (Abbott, Queensborough, UK)
- EMEM, (Lonza, Belgium).
- Phosphate Buffered Saline (PBS; Gibco, UK)
- HEPES (Lonza, Belgium)
- Collagenase P (Roche, UK).
- Heparin (1000 U/ml; Multiparin, Holder CP Pharmaceuticals Ltd., Wrexham, UK).

2.1.3. Cell Culture Media

- Heat- inactivated fetal calf serum (FCS; Invitrogen, Paisley, UK).
- Phenol red free William's E medium (WEM; Gibco; Life Technologies, UK).
- L-glutamine (2mM; Sigma-Aldrich Ltd., UK)

- Penicillin (50 U/mL; Sigma-Aldrich Ltd., UK)
- Streptomycin (50 mg/mL; Sigma-Aldrich Ltd., UK).
- Insulin-transferrin-selenium (ITS; Gibco Life Technologies, UK).
- Dexamethasone (Sigma-Aldrich Ltd., UK)

2.1.4. Cell Encapsulation

- NovaMatrix SLG20 ultrapure sodium alginate (FMC BioPolymer AS, NovaMatrix, Norway)
- Encapsulator (Model: IE-50R; Inotech Encapsulation AG, Switzerland)
- Saline solution (0.9% sodium chloride solution; Baxter Healthcare Ltd., UK)
- Calcium chloride ($CaCl_2$)(Sigma-Aldrich Ltd., UK),
- Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma-Aldrich Ltd., UK)
- Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS; Gibco, Life Technologies Corporation, UK)

2.1.5. Cryopreservation Solutions and Additives

Seven different commercially available cryopreservation solutions are used: ***Cell Banker 1*** (Nippon Zenyaku Kogyo Co., Ltd, Japan). ***Nutri Freez***^{TM} D10 (Sartorius, BI,USA), ***TC-Protector*** (Bio-Rad Laboratories Inc, USA), ***pZever*** (Sigma-Aldrich Ltd., UK), ***CP1*** (Koyoto Pharmaceutical Industry, Koyoto Japan), ***Cryo-JIN*** (REVIVE ORGANTECH Inc, CA, USA) and ***University of Wisconsin solution*** (***UW;*** Bristol-Myers Squibb AB, Sweden). Other conditions were tested using UW solution with other additive such as; ***Glucose*** 50% (Hameln pharmaceuticals ltd, UK), ***Dimethyl sulfoxide*** (DMSO; Sigma-Aldrich Ltd., UK), ***Trehalose*** (Sigma-Aldrich Ltd., UK), ***Z-VAD*** (Promega., UK), ***Ethylene Glycol*** (Alfa Aesar, Thermo Fisher Scientific, UK) and Polyvinylpyrrolidone (***PVP***; Sigma-Aldrich Ltd., USA).

Slow Rate Cryopreservation processes were carried out using Controlled-Rate Freezer (CRF; model: Kryo10, Planer, UK). And vitrification was done by using Liquid nitrogen.

2.1.6. Chemicals and Solutions for Hepatocyte Culture Assays

Ammonium chloride (NH_4Cl), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Omeprazole, Dexamethasone, ß-Naphthoflavone, Ethoxy resorufin salt, Resorufin, Salicylamide, Sulforhodamine B (SRB), Tris-base, Tri-chloroacetic acid (TCA), and Acetic acid (Sigma-Aldrich Ltd., UK).

2.1.7. Microbeads Cell Viability - Chemicals

- Fluorescein diacetate (FDA; Sigma-Aldrich Ltd., UK)
- Propidium iodide solution (PI; Sigma-Aldrich Ltd., UK)
- Vectashield antifade mounting medium (Vector Laboratories Inc., USA).

2.1.8. Assays and Cell Lysis Kits

- Rat, and Human Albumin ELISA Quantification kits (Bethyl Laboratories, USA).
- BioVision Urea Colorimetric Assay Kit (BioVision Incorporated, USA).
- Mammalian Lysis Kit (Sigma-Aldrich Ltd., UK).
- Bio-Rad DC™ Protein Assay (Bio-Rad).

2.1.9. Preparation of Solutions

- **1.5 % Sodium alginate solution**: Dissolve 1.5 g of sodium alginate in 100 ml of saline solution (0.9% NaCl; Baxter Healthcare Ltd., Norfolk, UK) using a stirrer.

- **1.2% Calcium chloride solution** (polymerization solution): 1.2 g $CaCl_2$ dissolved in 100 ml saline, then filtered through 0.2μm filter.
- **FDA solution**: 5mg FDA were dissolved in 1ml DMSO, stored at -20°C freezer until required.
- **50 mM Ammonium chloride (NH_4Cl; 10X)**: 2.65 mg of NH_4Cl was dissolved in 1 ml plain WEM.
- **Hepatocyte culture medium**: Hepatocytes where cultured in plates coated with 40% rat tail collagen, using William's E medium(Gibco; Life Technologies,UK) supplemented with 10% FCS (Invitrogen, Paisley, UK), 10-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Lonza), 2-mM L-glutamine, 0.1-mM dexamethasone (Sigma-Aldrich Ltd., UK), 0.1-mM insulin (ITS; Gibco, Life Technologies, UK);, penicillin (50 U/mL; Sigma-Aldrich Ltd., UK), and streptomycin (50 mg/mL; Sigma-Aldrich Ltd., UK).

2.1.10. Equipment & instruments

- Encapsulator (Model: IE-50R; Inotech Encapsulation AG, Switzerland).
- Water bath (Grant, JB Academy, UK).
- Plate reader (FLUOstar Omega plate reader, BMG LABTECH, UK)
- QuantaStudio 5 Real Time PCR (Thermo fisher scientific, USA).
- NanoDrop (ND-1000 Spectrophotometer, Thermo fisher scientific, USA).
- ABI microscope (EVOUS FL, Invitrogen by Thermo fisher scientific, USA).
- Controlled-Rate Freezer (CRF; model: Kryo10 from Planer, UK).
- Incubator (FisherBrand, Thermo fisher scientific, USA).
- Centrifuge (ROTINA 420R, Hittich, UK)

2.2. Methods

2.2.1. Hepatocyte Isolation

Human Hepatocytes isolation: Hepatocytes were isolated from donor livers unused for transplantation. All donor liver tissues were obtained through NHSBT and consented for research. Hepatocyte isolation and transplantation is carried out under Local Research Ethics Committee approval (King's College Hospital). The perfusion was done by using collagenase perfusion technique according to Mitry and colleagues (2009).

Major vessels were cannulated for the perfusion, Isolation solution were prepared in sterile conditions, the perfusion system was prepared, and oxygenation was supplied during the perfusion. First the liver tissue was perfused with (calcium free HBSS, 1M HEPES and 0.5M EGTA), then perfused with plane calcium free HBSS. Followed by perfusion with EMEM containing 0.05% collagenase P. Collagenase solution was re-circulated till good digestion occurred. After digestion completion cannula and sutures were removed and tissue is placed in ice-cold EMEM and minced using sterile scissors. Cells suspension was filtered with two layers sterile gauze. Hepatocytes were washed three times with ice-cold EMEM and centrifuged at 50xg, 4°Cfor 5min. Estimation of total cell number and viability were done using trypan blue exclusion test (Mitry,2009).

Rat Hepatocytes: Hepatocytes were isolated from Sprague- Dawley male rats (Harlan Olec, Bicester, UK) livers. Rats 8-10 weeks old and weight between 250-280 g were used and allowed to acclimatize at (room temperature 21± 2 °C & humidity 55± 10%) for one week before procedure. Proper protocols approved by the ethical board, King's College London in accordance with the UK Animals (Scientific) Procedures Act of 1986 were followed. Briefly, rats were anaesthetized using isoflurane, followed by abdominal incision to expose the liver. 100µl of heparin (1000 U/ml; Multiparin, Holder CP Pharmaceuticals Ltd., Wrexham, UK)

into inferior vena cava (IVC). The liver was then perfused with 250 ml PBS through the portal vein using an 18G needle and a perfusion pump, followed by 250 ml of EMEM/collagenase P solution to digest the tissue. The digested tissue was minced and ice-cold EMEM was added to inhibit the collagenase activity, followed by filtration through sterile gauze to eliminate cell clumps and undigested bits of tissue. Hepatocytes were washed three times with ice-cold EMEM and centrifuged at 50xg, 4°C for 5 min.

2.2.2. Cell Number and Viability

Cell number and viability of isolated hepatocytes were determined using standard trypan blue exclusion test. Equal volumes of diluted cells in EMEM and trypan blue were mixed. Each of the two hemocytometer chambers was loaded with 10 µl of cell/dye mix. Cell viability and total cell count were estimated.

2.2.3. Hepatocyte Culture

Hepatocytes were plated onto 40% collagen-coated plates, 200µl WEM supplemented with 10% FCS, 10 mM HEPES, 2mM L-glutamine, 0.1µM Dexamethasone, 5 ml ITS, penicillin 50 U/ml and streptomycin 50 ug/ml, at 50,000 cells/well in (96-well plates for MTT and SRB assays), and 250,000 cells/well in 500µl WEM (24-well plates for albumin, urea, and CYP P450 assays). Medium was changed as appropriate according to the time point and the assay. Hepatocytes were cultured in a humidified incubator at 37°C and 5% CO_2.

2.2.4. Hepatocyte Overall Metabolic Activity – MTT Assay

The overall metabolic activity of the hepatocyte was assessed using the MTT Assay. Working solution of MTT was prepared by dissolving MTT stock solution (5mg/ml in PBS) in plain WEM (ratio 1:10). The isolated hepatocyte were cultured in in 200ul WEM in 96- well plate and after 24, 48, 72h the cells were washed with sterile PBS followed by addition of 200

µl of diluted MTT/well. The plate was re-incubated at 37°C, 5% CO_2 for 4 hours then supernatant was aspirated from wells. 200µl of DMSO was added to each well and plate was covered and left for 10 min on a plate shaker at high speed. The absorbance was read at 550 nm using the FLUOstar Omega plate reader.

2.2.5. Cell Attachment - Sulphorhodamine B (SRB) Assay

Hepatocyte were cultured in 40% collagen-coated 96-well plates. After 24, 48, 72h, media was aspirated from wells followed by one wash with PBS. 200 µl of serum-free WEM was added to each well followed by 50µl of ice cold 50% TCA and plate was incubated for 1h at 4°C to fix cells. Wells were rinsed 5x with tap water, then cells were stained with 50 µl of 0.4% SRB at room temperature for 10 min. SRB was aspirated and wells were rinsed 5x with 1% acetic acid and left to air dry at room temperature. SRB dye was solubilized by adding 200 µl of unbuffered Tris-base. Absorbance was measured at 564 nm using FLUOstar Omega plate reader.

2.2.6. Albumin ELISA Assay

Isolated hepatocytes were cultured in 96- well plate and after 24, 48, and 72h the supernatant was collected for albumin quantification. Rat, and Human Albumin ELISA Quantification Kits (Bethyl Laboratories, USA) were used to quantify the amount of serum albumin produced by the rat and human hepatocytes according to the manufacturer protocols.

Briefly, 100 µl of diluted coating anti-albumin antibody (anti-rat or anti-human albumin) were placed in each well of high binding 96-well plate (Microlon®; Greiner Bio-One GmbH, Germany). Plate was incubated at room temperature for one hour, then wells were washed 5x with wash buffer, followed by placing 200 µl of blocking solution in each well and incubation for 30 min at room temperature. Wells were then washed 5x with wash buffer. Serial dilution of rat or human albumin reference were prepared (0-400 ng/ml). Duplicates of 100 µl

of each sample and standards were placed in wells, following 1h incubation at room temperature, wells were wash 5x with wash buffer. 100 µl of diluted HRP detection antibody was then placed in each well and plate was re-incubated for 1h. Wells were washed 5x with wash buffer, and 100 µl of TMB substrate solution were placed in each well and allowed to develop in dark conditions at room temperature for 15min. The reaction was stopped by adding 100 µl of 2M sulphuric acid solution (stop solution) to each well. Absorbance was measured at 450 nm using FLUOstar Omega plate reader, and albumin was quantified using the albumin standard curve.

2.2.7. Hepatocyte Ammonia detoxification - Urea Assay

Urea syntheses were induced by adding (1 mM) of ammonium chloride for the hepatocytes culture and supernatants were collected for each time 24, 48 and 72 hours. BioVision urea colorimetric Assay Kit was used to measure the amount of urea secreted by the hepatocyte. Serial Dilution of standard urea (50 mg/dl) were prepared using distilled water. 25μl of the sample buffer and 25 μl of each standard and samples were added to each will, then reagent mix was prepared and 50 μl added to each well. O.D reading at 570nm was performed, urea concentration of the samples then calculated referring to the standard concentration.

2.2.8. Cytochrome P450 EROD Assay (CYP 450 A1/2)

Cytochrome P450 was measured by ethoxy resorufin-O-demethylase (EROD) activity, EROD was prepared by mixing 2mM of Ethoxy resorufin (7-ER) and 150 mM of salicylamide.

CYP 450 was induced in cultured human hepatocyte using Omeprazole, while in rat hepatocytes was induced using β-Naphthoflavone, for 24, 48 and 72h. EROD was added to treated cultures, while control cultures were treated with DMSO. In this assay cell culture medium used was without Dexamethasone. Serial dilutions of Resorufin (10mg/ml) standards (0-100ng/ml) were prepared. The florescence of resorufin standards and samples were read at

excitation of 530 nm/emission of 590 nm using FLUOstar Omega plate reader, and the resorufin content of the hepatocyte cultures were estimated.

2.2.9. Total Cell Lysate

Total cell lysates of cultured hepatocytes were prepared at the end of each time point using Mammalian Cell Lysis Kit (Sigma-Aldrich Ltd., UK) and according to manufacturer's protocol. Briefly, wells were washed with PBS (500µl/well), then 100µl of cell lysis and protein solubilisation buffer (Tris-EDTA, NaCl, SDS, DOC, Igepal CA360, and protease inhibitor cocktail) were placed in each well.

Plate(s) was/were placed on an orbital plate shaker for 15min, then cell scraper was used to scrape of cells. Crude cell lysates were collected in appropriately labelled 1.5ml microfuge tubes and centrifuged at 12,000xg, 4°C for 10min to pellet cell debris. Supernatants containing protein were transferred into fresh appropriately labelled 1.5ml microfuge tubes and either maintained on ice or stored at -20°C until protein content quantification was carried out.

2.2.10. Protein Content Quantification

Bio-Rad DC Protein Assay kit was used to measure the protein content of total cell lysates, according to manufacturer's protocol. Briefly, duplicates of 5µl samples of total cell lysate were placed in wells of a 96-well plate. 20 µl of reagent S was added to one ml of reagent A. Bovine serum albumin (BSA) standards (0.2 – 1.5 mg/ml) were prepared by diluting a 10 mg/ml BSA stock solution. 50s µl of each standard and samples were placed in each well. Then 50 µl of reagent mix was added to each well and incubated for 15 min, absorbance was done at 750nm using FLUOstar Omega plate reader. The protein content of each sample was estimated using the BSA standard curve.

2.2.11. Cryopreservation & Vitrification

2.2.11.1. Preparation of cryopreservation solution

Ready-made cryopreservation solutions from different suppliers, with different ingredients and cryoprotectant(s)were used to test their effects on hepatocyte cryopreservation. All tested solutions were compared to UW solution supplemented with 10% DMSO and 5% glucose. 5 ml cryo-tubes were used and a final cell density of 1 $x10^7$ cells/ml was maintained in all experiments.

2.2.11.2. Hepatocyte cryopreservation

Cryopreservation processes were carried out using Controlled-Rate Freezer (CRF; model: Kryo10, Planer, UK).

2.2.11.3. HMBs Vitrification

Vitrification was carried out on HMBs in 50 ml cryo-bags by immediate immersion in liquid nitrogen, then bags were transferred to -140ºC freezer. Each bag contained 10 ml of HMBs suspension in cryopreservation solution.

2.2.12. Hepatocyte Encapsulation

2.2.12.1. Preparation of hepatocyte/alginate mixture

Sodium alginate (1.5%) was prepared by adding 16.6 ml saline solution to one bottle of sodium alginate (250mg). The alginate was dissolved overnight on an orbital shaker. Hepatocytes were slowly added to the alginate solution at a density of $2.5x10^6$ cells/ml, then the hepatocyte/alginate mixture was transferred into a 50 ml syringe fitted with a Kwill®.

2.2.12.2 Encapsulation run

The hepatocyte encapsulation protocol used was developed by Dr. Mitry at the Institute of Liver Studies, King's College Hospital (Mitry et al., 2016) and with some modifications.

Briefly, using the Inotech encapsulator IE-50R fitted with a 250µm nozzle; the following parameters: vibration frequency at 1300 Hz, electric charge at 80-90V, and amplitude at 4. The electrode ring and bypass cup was placed correctly. 250ml polymerization solution (1.2% $CaCl_2$ in 0.9% saline; w/v) were placed in the reaction vessel. The 50 ml syringe containing the hepatocyte/alginate mixture was placed in the syringe pump. The pump was started and was run at a speed setting of 300 (6.7 ml/min). Polymerized microbeads were left in the polymerization solution for 10min with continuous gentle stirring, then microbeads were washed 2x with 0.9% saline (each wash for 7 min). At the end of the second wash, the microbeads were collected and washed 1x with plain WEM medium.

2.2.13. Microbeads Culture

Microbeads were cultured in 24-well plates in culture medium consisting of WEM supplemented with 10% FCS, 10 mM HEPES, 2mM L-glutamine, 0.1µM Dexamethasone, 5 ml ITS, penicillin 50 U/ml and streptomycin 50 ug/ml. The Microbeads cultures were maintained in a humidified incubator at 37°C and 5% CO_2 and tested at various time points depending on the experiment.

2.2.14. Hepatocyte Microbeads MTT Assay - Overall Metabolic Activity

The overall metabolic activity of encapsulated cells was determined using the MTT Assay. Briefly, MTT solution 5mg/ml in PBS were diluted in WEM 1:10. 250 µl of microbeads were placed in a 1.5 ml microfuge tube, 500 µl of diluted MTT were added and tubes were incubated at 37°C for 4 hours. Microbeads were washed with 500µl DPBS, and 500µl of DMSO were placed. Tubes were left for 10 min on vigorous shaking, then tubes were centrifuged at high speed, and supernatants were collected. 200 µl supernatant per well of a 96-well plate were placed (duplicates). The optical density readings at 550 nm were obtained using the FLUOstar Omega plate reader.

2.2.15. Hepatocyte Microbeads Albumin Assay

Human Albumin ELISA Quantification Kit were used to quantify the albumin secreted by the encapsulated hepatocytes. The supernatant was collected from encapsulated microbeads for albumin quantification. 96-well plates (C96 Nunc) were coated with anti-human albumin antibody for 1 hour, then washed with wash solution (0.05% Tween 20% Tris –Buffered saline) after that blocked with 1% BSA/Tris buffered saline for 30 min. Serial dilutions of Albumin Standard were prepared, Duplicates of each standard or samples were added to the wells, followed by addition of diluted HRP detection antibody and incubated for one hour, then washed 5x with wash solution. After that TMB substrate solution was added and the plate was left at dark for 15min, then reaction was stopped by adding the stop reaction solution. Finally, absorbance was measured using microplate reader (FLUOstar Omega) at wavelength 450 nm.

2.2.16. Hepatocyte Microbeads Urea Assay

Urea syntheses were induced by adding (1 mM) of ammonium chloride for the microbeads culture. supernatants were collected for day 1, day 3 and day 7. BioVision urea Colorimetric Assay Kit was used to measure the amount of urea secreted by the hepatocyte. Serial dilutions of standard urea (50 mg/dl) were prepared using molecular biology grade water. 25µl of the sample buffer and 25 µl of each standard or samples were placed in each well, then reaction mixture was prepared and 50 µl added to each well. O.D reading at 570nm was performed, urea concentration of the samples then calculated using the standard curve.

2.2.17. HMBs Cytochrome P450 EROD Assay (CYP 450 A1/2)

Cytochrome P450 activity was measured using ethoxy resorufin-O-demethylase (EROD) assay in which a mixture of2mM of Ethoxy resorufin (7-ER) and 150 mM of salicylamide was used.

Human Hepatocyte microbeads were induced by Omeprazole, while rat hepatocyte microbeads were induced by β-Naphthoflavone, from day 1 till day 7 respectively. EROD was added to treated samples, where control samples were treated by DMSO. In this assay cell culture medium used was without Dexamethasone. Serial dilutions of Resorufin (10mg/ml) standards (0-100ng/ml) were prepared. The florescence of resorufin standards and samples were read at excitation of 530 nm/emission of 590 nm using FLUOstar Omega plate reader.

2.2.18. Cells Viability Test Using FDA/PI

Microbeads were stained with FDA/PI; 10ul of 1mg/ml FDA and 20ul of 1mg/ml PI in dark condition for 90 sec and washed two times with DPBS and re-suspended at 250µl DPBS. A sample of stained microbeads were placed on a glass microscope slide. Cells were visualized under fluorescent microscope. Live cells cytoplasm stained green (FDA) and the nuclei of dead cells stained red (PI). Random images were taken for qualitative assessment of cell viability within the microbeads.

2.2.19. RNA Extraction

Cryopreserved hepatocytes were thawed and cultured for 24h, RNA extractions were conducted by using Direct-zol^{TM} RNA Mini prep extraction kit (ZAYMO Research, USA), Average of one million living cell where cultured in 6 well plate, for 24h, cells were washed with BPS and treated with 300 µl TRIzol® Reagent (Ambion, life technologies, UK). Equal volume of 100% Ethanol added to the sample and mixed thoroughly, mixture then transferred to Zymo-spin column and collection tube followed by centrifugation at 10,000 Xg for one min. The column was washed with 400 µl of RNA wash buffer, followed by centrifugation for 1min at 10000Xg. Mix of 5µl of DNase I and 75 µl of digestion buffer were added to digest any leftover of DNA. Then 400µl of Direct-Zol^{TM} prewash was added followed by centrifugation

at 10,000 Xg for one min. after that 700µl of wash buffer was added to each column and centrifugation at 10,000 Xg for one min. then Column was transferred to new RNase free collection tube and 50 µl of DNase/RNase free water was added to elute the RNA followed by centrifugation at 10,000 Xg for one min.

2.2.20. RNA Quantification

RNA quantification was conducted using Nanodrop and 1 µl of RNA sample. RNA concentrations measured had a range of 90-250 ng/µl (appendix). Samples were stored at -80^{o}C until needed.

2.2.21. Reverse Transcription-Polymerase Chain Reaction

Reverse-transcription (RT) were conducted to produce cDNA using Qiagen Omniscript® RT Kit (Qiagen, Germany). 2µg of template RNA was used in a 40 µl PCR reaction [4µl 10X buffer, 4 µl dNTPs mix (5 mM each), 2µl Oligo-dT Primer (10 µM), 1 µl RNase inhibitor (40 units/µl) , 2 µl Omniscript Reverse Transcriptase], and each reaction volume was topped up to 40 µl using RNase-Free water. Reactions were carried out in 0.5µl Eppendorf tubes, and incubation at 37C^{o} For 60 min in an incubator. At the end of the reaction, tubes were allowed to acquire room temperature then stored at -20C^{o} until needed for real-time PCR runs.

2.2.22. TaqMan Gen expression Assay

Relative quantification Real-Time PCR were carried out using ready-made TaqMan® Gene Expression Assays, and 384-well PCR plate. 20µl PCR reaction per sample was prepared by using 2 µl of cDNA template, 10 µl TagMan™ Universal Master Mix II no UNG, total volume where toped up by 8 ul RNase free water, Using 384 PCR Plate and QuantaStudio 5

Real Time PCR. Each PCR run was carried out at 95°C for 10min (Hold step), followed by 40 cycles of 95°C for 15 sec (denaturation), and 60°C for 1 min (annealing/extension). Data obtained were analysed using Relative Quantification method, and QuantStudio™ Design & Analysis Software (ver. 1.5.1 Applied Biosystems, Thermo Fisher Scientific).

2.3. Statistical Analysis

Statistical analyses were cried out using GraphPad Prism 7 Software. The data is expressed as the mean ± standard deviation (SD) and n is mentioned in text accompanying the figure. Student t-test was used to compare between two values. Groups with more than 3 values were analysed using two-way analysis of variance (ANOVA) and multiple comparisons were adjusted by using the Tukey's post-hoc test. $P \leq 0.05$ was considered significant.

Chapter 3

Hepatocyte Isolation & Culture

3.1. Introduction

3.1.1. Hepatocyte isolation

Hepatocytes are a vital tool for liver-related research, isolated hepatocytes are currently used by many research groups studying on liver physiology, drug testing, BAL systems, and many clinical applications mainly transplantation in patients with liver disease. Therefore, isolating good quality hepatocyte from liver tissues unused/rejected donor for transplantation is the key to continue such research.

The first rodent and human hepatocyte isolation protocol was developed in late 1960s by Berry and Friend, and later in 1982 Guguen-Guillouzo et al, published the first isolation protocol of human primary hepatocyte using two step prefusion method which is well known as collagenase perfusion protocol (Green et al., 2017), which resulted in high quality hepatocytes with good viability (Larusso, 1994).

At King's College, the Hepatocyte Biology & Transplantation team using a protocol for hepatocyte isolation that they have successfully modified (Terry et al., 2006).

3.1.2. Hepatocyte viability and activity

Determination of isolated hepatocyte viability and activity is very important to assist the quality of the isolated hepatocyte, in this chapter , collagenase prefusion protocol have been used to isolate rat and human hepatocyte, isolated hepatocyte then cultured and assessed for its viability and activity using different assays such as overall hepatocyte activity using MTT

assay, attachment activity SRB assay, albumin synthesis, Urea production, and Detoxification as indicated in the following sections.

3.2. Materials and Methods

3.2.1. Materials

3.2.1.1. Human and rat hepatocyte isolation solutions

Solutions used in the hepatocyte isolation protocols described in chapter 2 Section (2.1.1 & 2.1.2) respectively.

3.2.1.2. Hepatocyte Culture

hepatocyte where cultured in plates coated with 40% rat tail collagen, Phenol red free William's E medium (WEM) where used supplemented with; heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, penicillin/streptomycin, and insulin-transferrin-selenium (ITS) and dexamethasone. Hepatocytes were cultured in a humidified incubator at 37°C and 5% CO_2, media were changed every 24, 48, 72h depending on the experiments set.

3.2.1.3. Hepatocytes Assays chemicals and kits

All kits and assays used to determine hepatocyte viability and activity are described in Chapter 2.

3.2.2. Methods

3.2.2.1. Hepatocyte Isolation

Human hepatocytes were isolated from donor livers unused for transplantation, and rat livers using protocol mentioned in Chapter 2 Section (2.2.1).

3.2.2.2. Cell Number and Viability

Cell number and viability were determined using trypan blue exclusion test Chapter 2, Section (2.2.2).

3.2.2.3. Hepatocyte Culture

Hepatocytes were plated in collagen-coated plates, different types of culture plates were used depending on the assay(s), (**Table 3.1)**. Culture medium was changed as appropriate according to the time point of assays. Plates were incubated in a humidified incubator at 37°C and 5% CO_2.

Table 3.1: Hepatocyte Culture Plates

Plate type	Media volume	Cell Density	Assays
96-well plate	200µl WEM	50,000 cells/well	- MTT - SRB
24-well plate	500µl WEM	250,000 cells/well	- Albumin estimation - Urea - CYP P450 assay

3.2.2.4. Hepatocyte's Activity Assays

Hepatocyte Overall Metabolic Activity **al assays were** conducted as described in **chapter 2; (MTT Assay;** Section: 2.2.4**),** Cell Attachment – Sulphorhodamine B **(SRB Assay** Section; 2.2.5**),** Albumin (**ELISA Assay;** Sections 2.2.6**),** Ammonia detoxification (**Urea Assay;** Section 2.2.7), Cytochrome P450 (**EROD Assay**, **CYP 450 A1/2;** Section 2.2.8).

3.2.2.5. Total Cell Lysat and Protein Content Quantification

Protein content quantification was carried out using Bio-Rad DC™ Protein Assay kit to measure the protein content of total cell lysates, according to manufacturer's protocol described in Chapter 2, Sections 2.2.9 and 2.2.10.

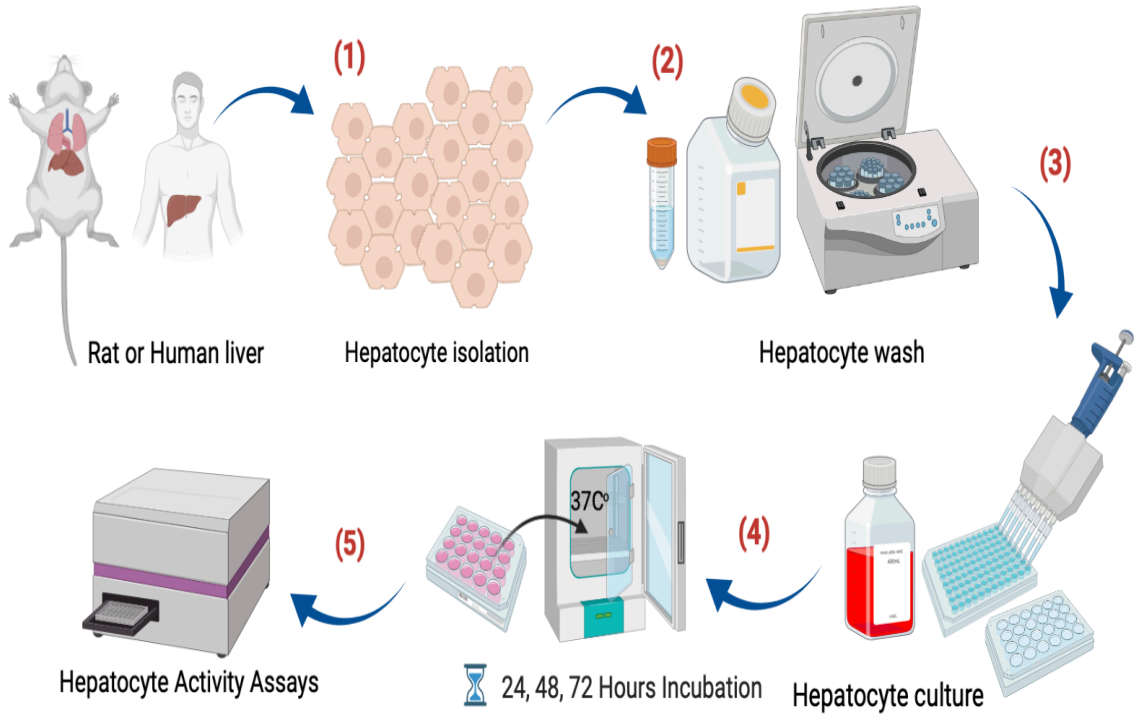


Figure 3.1: Hepatocyte Isolation and Culturing

[Illustration created using BioRender platform, BioRender.com]

3.3. Results

3.3.1. Hepatocyte Isolation

Human and rat hepatocyte were isolated using collagenase prefusion protocol. Un used human liver for transplantation were used for hepatocyte isolation, and Sprague- Dawley male rats' livers were used for hepatocytes isolation as shown in figures bellow.

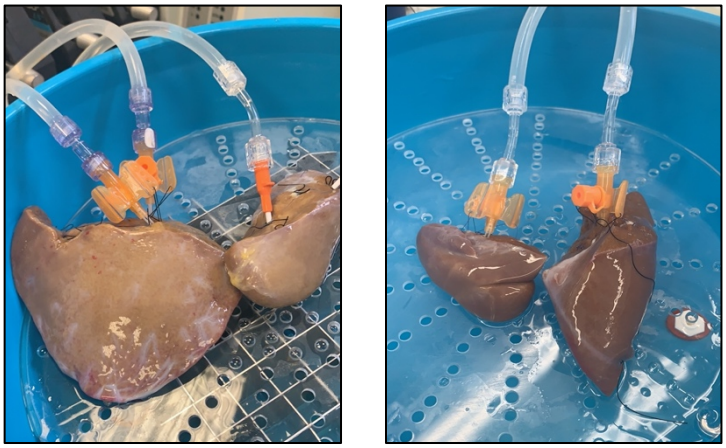


Figure 3.2: Human hepatocyte isolation; unused liver for transplantation were used for hepatocyte isolation, using collagenase prefusion protocol.

Figure 3.3: Rat hepatocyte isolation; Sprague- Dawley male rats (Harlan Olec, Bicester, UK) livers were used for hepatocyte isolation (rats: age,8-10 weeks old; weight, 250 - 280 g).

3.3.2. Hepatocyte Viability Determination Using Trypan Blue Exclusion

After hepatocyte isolation, human and rat hepatocyte viability were determined using trypan blue exclusion. Average viability between 75% - 85% were obtained using the collagenase profusion hepatocyte isolation protocol.

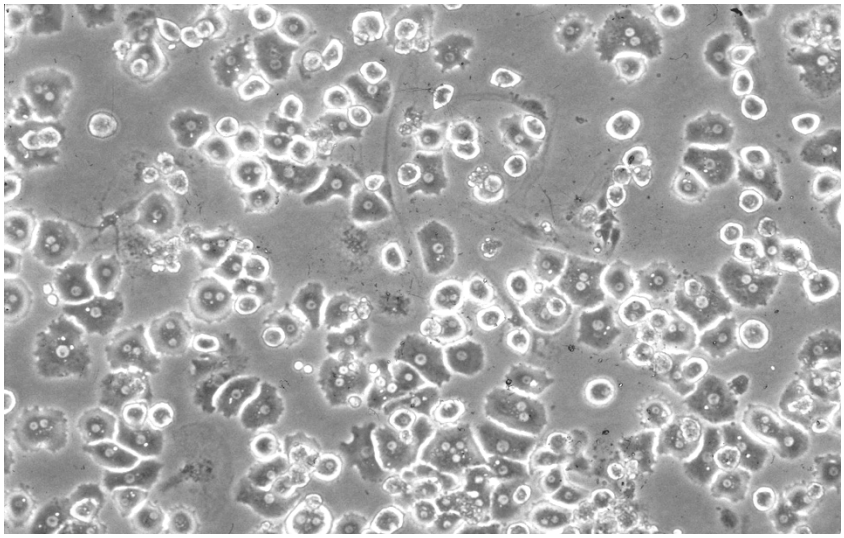


Figure 3.4: Human hepatocyte culture 24h post plating. [objective: 10X]

3.3.3. Fresh Hepatocyte Overall Metabolic Activity -MTT Assay

Hepatocyte overall metabolic activity tested at 24, 48 and 72 hours of fresh rat hepatocyte **Figure (3.5A),** showed that cultured hepatocytes maintained its overall activity at all time points with decreasing in their activity by time.

3.3.4. Fresh Hepatocyte Attachment Activity- Sulphorhodamine B (SRB) Assay

Cell to cell contact and cell adhesion to extracellular matrix determines cell activity and function, here hepatocyte attachment efficiency is determined by its ability to attaches to the culturing plate through the SRB assay, freshly cultured rat hepatocytes were tested for their ability to adhere to the culture plate. Cell attachment of the fresh hepatocyte tested at 24, 48 and 72 hours, **Figure (3.5B)**, showed that cell were viable and able to adhere to culture plate, however, there was a progressive decrease in fresh hepatocytes attachment level with time.

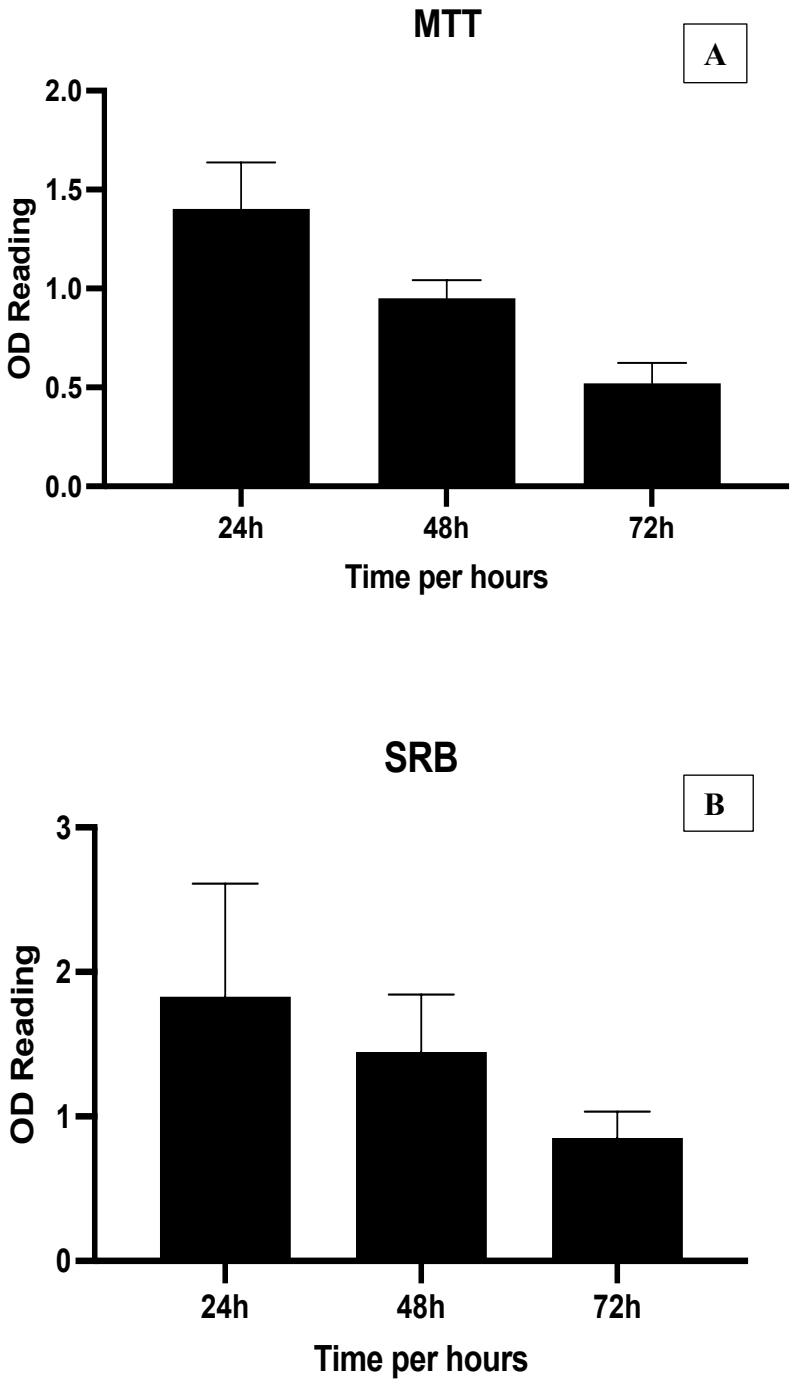


Figure 3.5: A) MTT results of overall activity of fresh cultured rat hepatocytes, **B) SRB** results of fresh cultured rat hepatocytes attachment activity, Data are presented as mean ± SD, n=16.

3.3.5. Fresh Hepatocyte Ammonia Detoxification - Urea Assay

Urea production is another hepatocyte-specific function that is based on detoxification of ammonia, this process is mediated through urea cycle within the hepatocyte, the ability of induced cultured hepatocyte to produce urea determine its ability to detoxify the ammonia. This test was carried out by challenging hepatocytes with ammonium chloride compared to control culture (unchallenged). Data presented in **Figure (3.6)** shows that induced hepatocytes had higher urea production level compared to the control hepatocytes.

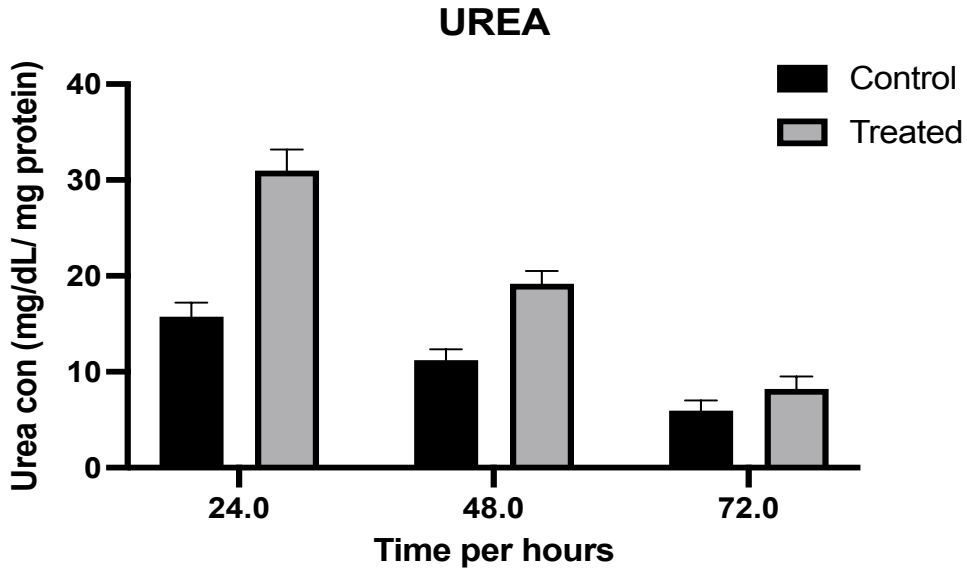


Figure 3.6: Urea assay results following rat hepatocyte challenge with ammonium chloride compared with un-challenged cells. Data are presented as mean ± SD, n=16.

3.3.6. Fresh Hepatocyte Albumin Production

Albumin is one of the most important proteins in the body, produced by hepatocytes in the liver. Functioning viable hepatocytes are able to synthesise albumin, therefore, to determine the activity of cultured hepatocyte, albumin was measured at different time points **Figure (3.7)**.

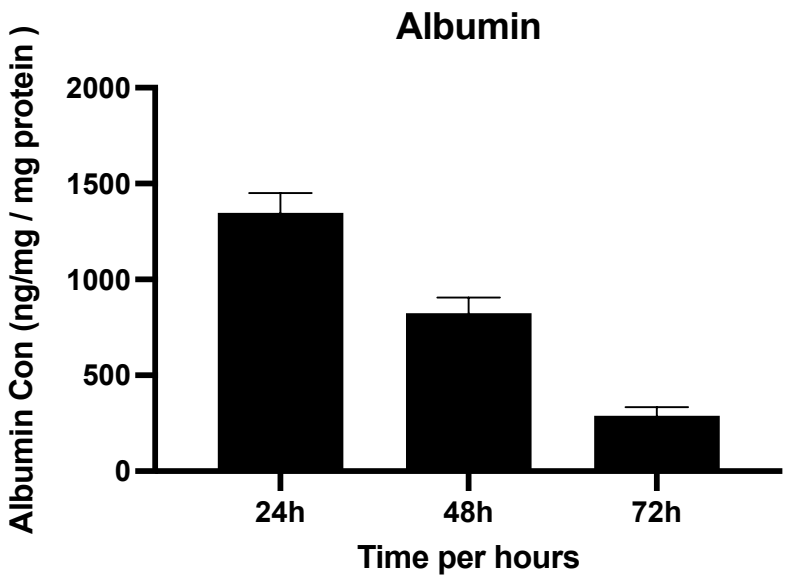


Figure 3.7: Albumin production of freshly cultured rat hepatocyte, hepatocytes were able to produce Albumin with a progressive decrease with time point. Data are presented as mean±SD, n=16.

3.3.7. Cytochrome P450 EROD Assay (CYP 450 A1/2)

Cytochrome P450 (CYP450) activity in the liver is one of the essential functions for drug metabolism and toxins detoxification. Ethoxy resorufin-O-demethylase (EROD) assay is used to determine the level of induction of the xenobiotic-metabolizing enzyme cytochrome P-450 (CYP) 1A1/2 during the detoxification process by hepatocytes. Human hepatocytes were induced by Omeprazole, while Rat hepatocyte were induced by β-Naphthoflavone, for 24, 48 and 72 hours, then florescence of resorufin standards and samples were read at excitation of 530 nm/emission of 590 nm using FLUOstar Omega plate reader. Results showed that cultured hepatocyte were able to detoxify the xenobiotic agent through the activation of CYP 450 metabolic enzymes, however the activity of hepatocyte were reduced by time. EROD data shown in **Figure (3.8)**.

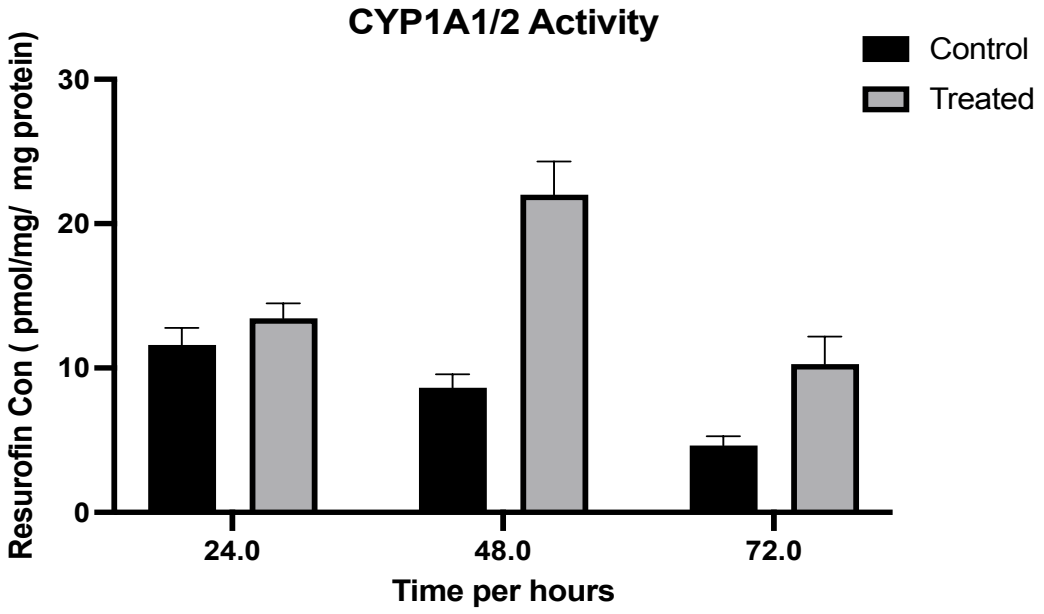


Figure 3.8: Cytochrome P450 activity in fresh induced rat hepatocyte cultures. Data are presented as mean ± SD, n=16.

3.4. Discussion and Conclusion

Hepatocyte isolation and culturing is the main tool to study the hepatocyte activity, function, drug metabolism, liver research and many clinical applications.

Hepatocyte isolation using the modified collagenase prefusion protocol of King's College London hepatocyte team; resulted in good quality of isolated hepatocyte.

Hepatocyte culture showed that isolated rat and human hepatocytes maintained their viability and function when cultured with William's E medium (WEM) supplemented with; heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, penicillin/streptomycin, and insulin-transferrin-selenium (ITS), and dexamethasone, were incubated in a humidified incubator at 37°C and 5% CO_2. Hepatocyte-specific assays were carried out every 24, 48, 72h respectively. Human and rat hepatocytes maintained their functions but showed reduced activities with time.

In conclusion "good" quality rat and human hepatocytes were isolated using collagenase prefusion protocol and tested *in vitro*, the isolated hepatocytes can be used in the next planned studies including cryopreservation, and encapsulation.

Chapter 4

Hepatocyte Encapsulation

4.1 Introduction

Encapsulation is a process of coating cells with semipermeable membrane to protect cells from host immune system (Nicodemus & Bryant, 2008). The encapsulation membrane should be permeable to allow solutes, nutrients, and oxygen exchange between cells. When choosing encapsulation material, many characteristics should be taken in consideration such as wall thickness, permeability of oxygen and nutrients, waste secretion, bio-commutability, and to provide similar in vivo conditions for cells and not cause any inflammation or allergy reaction to the host (Nicodemus & Bryant, 2008; Hunt & Grover, 2010). Alginate is a commonly used encapsulation material, due to its good permeability properties as well as biocompatibility (Leong et al., 2016). Alginate cell encapsulation have wide range of application in the, especially that alginate provides immune-isolation for the transplanted cells therefore will eases the transplantation without the need of immunosuppression medication (Young et al., 2012), **Figure (4.1)**.

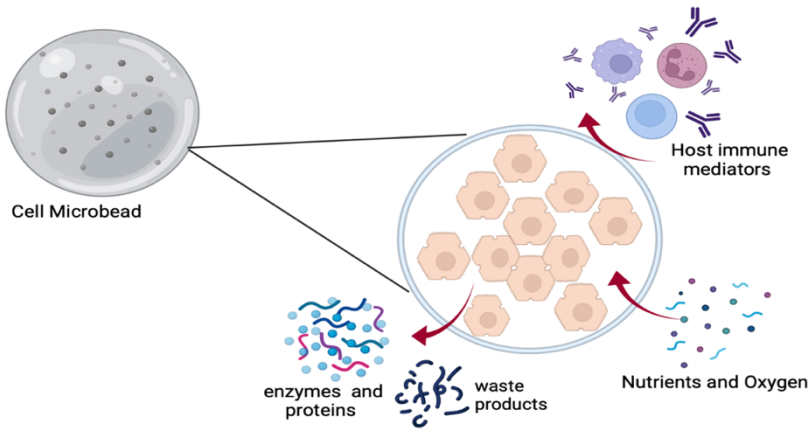


Figure 4.1: Cell Encapsulation, Permeability, and Immune Isolation

[Illustration created using BioRender platform, BioRender.com]

Different types of cells have been encapsulated for clinical and research application. Human hepatocyte encapsulation offered the chance for many patients with liver failure to survive longer, Hepatocyte transplantation team at King's College Hospital are world pioneers who started alginate-encapsulated human hepatocyte transplantation. The transplantation was successful and proved that encapsulated hepatocyte were capable to survive for 6 months in the peritoneal cavity of the patients without the need for immune suppression medications (Jitraruch et al., 2014).

In this chapter alginate SLG20 have been used for encapsulation of hepatocytes, by mixing the cells with 1.5% Alginate solution then using encapsulator model: Inotech R-50, were hepatocytes resuspended in alginate and placed in a 50ml syringe. The suspension was extruded as droplets into polymerisation solution (1.2% $CaCl_2$), where polymerisation of alginate takes place. Controlled vibration through the 250 μm nozzle and electro spraying by applying electrical potential around the tip of the nozzle with frequency 1300 Hz, amplitude at 4 and voltage 80-90 V, and syringe pump speed at 300, these parameters lead to the production of microbeads with a diameter of 500-700 μm.

4.2 Material and Methods

4.2.1 Encapsulator Settings

Different settings on IE-50R encapsulator were tested to get the optimum size and shape of alginate microbeads. empty microbeads were first produced to determine the optimal settings on the encapsulator, then hepatocyte microbeads were produced using these settings.

4.2.2 Hepatocyte Encapsulation

4.2.2.1 Preparation of Hepatocyte Alginate Mixture, and Polymerization Solution

Sodium alginate of 1.5% was prepared by adding 16.6 ml saline solution to one bottle of sodium alginate (250 mg). The alginate was dissolved overnight on an orbital shaker. Hepatocytes were slowly added to the alginate solution at a density of $2.5x10^6$ cells/ml, then hepatocyte/alginate mixture was collected in 50 ml syringe using a Kwill®. Then encapsulation protocol developed by Dr. Mitry at the Institute of Liver Studies, King's College Hospital (Mitry et al., 2016) was used with some modifications as mentioned in Chapter 2, Section (2.2.12).

4.2.2.2 Microbeads Culture

Microbeads were cultured in 24 well plates in a humidified incubator at 37°C and 5% CO_2 then tested for its activity on day1,3 and 7 repeatedly. Culture media used for microbeads culturing is mentioned in Chapter 2 Section (2.2.13).

4.2.3. Hepatocyte Microbeads Viability and Activity Assays

Hepatocyte microbeads activity were tested on Day 1, 3 & 7 for the following assays as described in Chapter 2; Overall Metabolic Activity MTT Assay Section (2.2.14), Albumin Assay Section (2.2.15), Urea Assay Section (2.2.16) and Cytochrome P450 EROD Assay (CYP 450 A1/2) Section (2.2.17).

4.2.4. Cell Viability Test Using FDA/PI

Hepatocyte viability within Microbeads were determined by using FDA/PI see Chapter 2, Section (2.2.18).

4.3. Results

4.3.1. Microbeads Size and Morphology Optimization Using SLG20 Alginate

The initial experiments of alginate encapsulations of hepatocytes were carried out to optimize the microbeads size as well as to optimize the encapsulator settings to produce "good" quality microbeads for transplantation.

4.3.2. Flow Rate Optimization

Flow rate was optimized using 1.5% SLG20 alginate with different pump speed, 100, 200, 300, 400 and 500 using 50 ml syringe and 250 µm nozzle **Table (4.1)**.

Table 4.1: Flow Rate Determination using 50ml Syringe and 1.5% SLG20 Alginate

Pump Speed	W1	W2	Flow Rate ml/min
100	3.6g	3.3g	3.45ml/min
200	5.6g	5.5g	5.55ml/min
300	10.6g	10.1g	10.35ml/min
400	11.5g	11.3g	11.4ml/min
500	13.3g	13.5g	13.4 ml/min

4.3.3. Microbeads Size Optimization with Different Frequency

Pump speeds were tested on different speeds to determine the optimum flow rate using 1.5% SLG20 Alginate and 250 µm nozzle. optimum size of microbeads was obtained when using; nozzle size of 250µm, frequency 1300 Hz, amplitude at 4 and voltage 80V, and syringe pump speed at 300. The average size of microbeads obtained when using the above-mentioned settings was 514.5 µm ± SD as shown in **Figure (4.2)** and **(4.3)**.

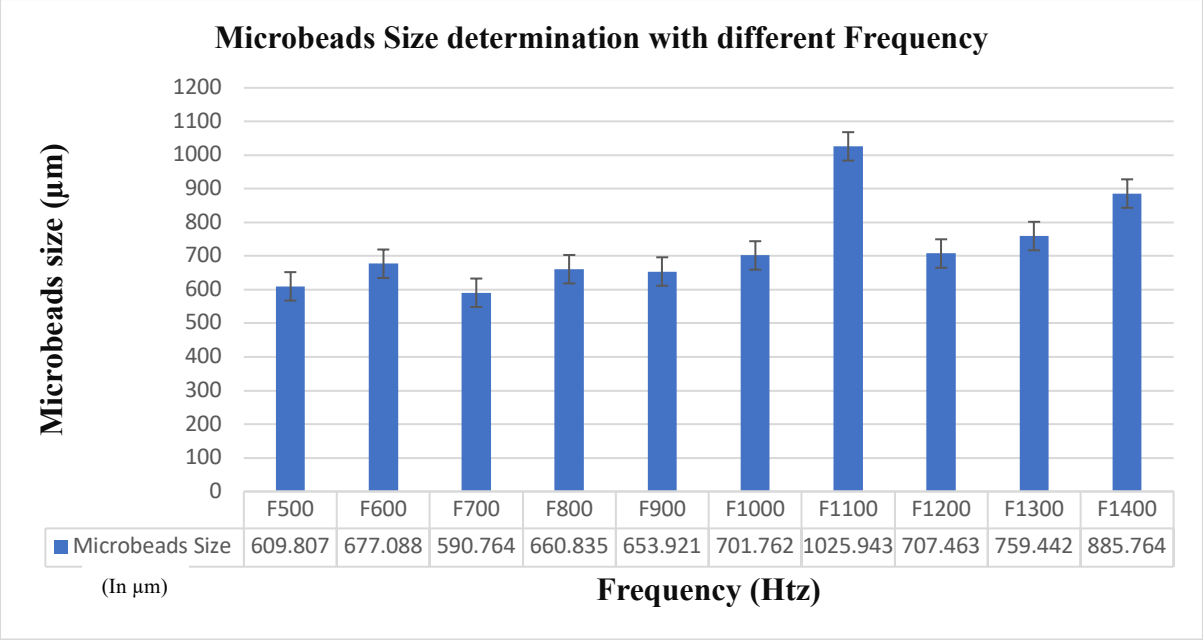


Figure 4.2: Microbeads Size Optimization with different Frequencies.

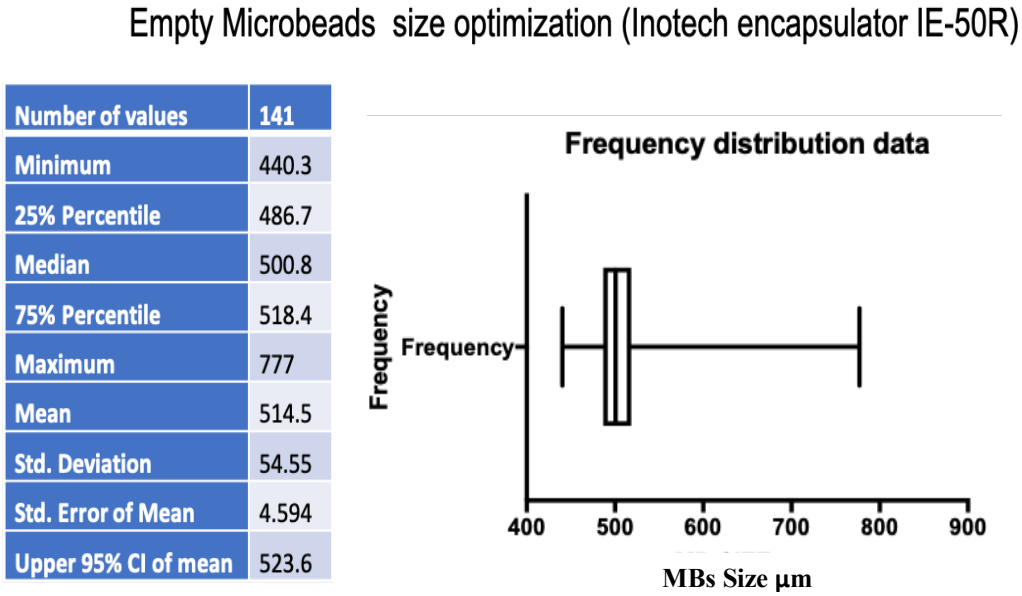


Figure 4.3: optimum size of Microbeads was obtained when using Frequency =1300Hz, Voltage =0.80kV, Amplitude = 4 and syringe pump speed = 300. The average size of microbeads using 250µm nozzle and above-mentioned settings was: mean 514.5 ± SD 54.55 µm.

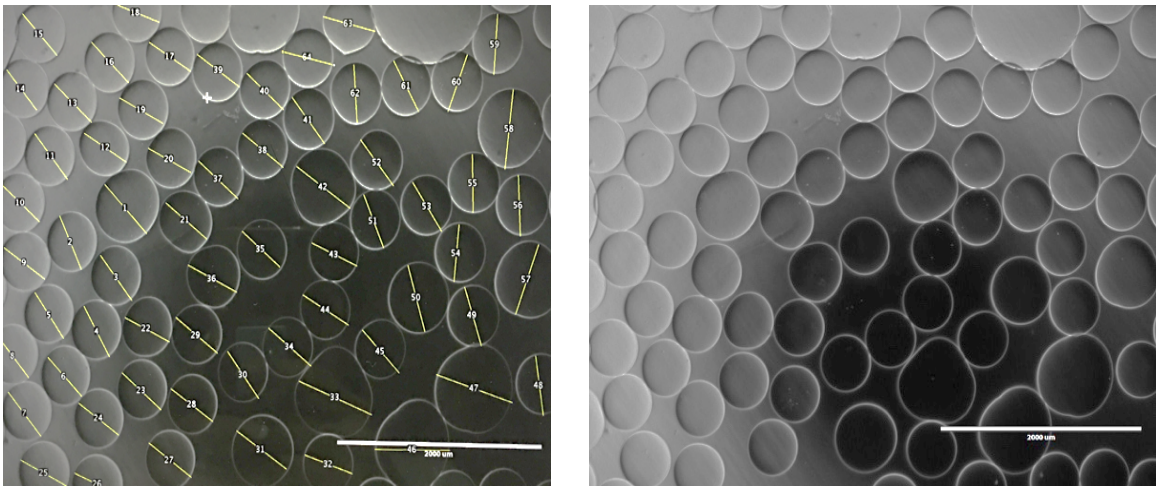


Figure 4.4: Empty microbeads were produced using Inotech encapsulator IE-50R fitted with a 250µm nozzle, pump speed 300 with frequency 1300 Hz, and voltages 0.8, The average microbead diameter measured using ImageJ software was 500-700 µm.

4.3.4. Production of Hepatocyte Microbeads

In 2014 Jitraruch and colleagues showed that a density of $2.5x10^6$ hepatocytes per ml of 1.5 % SLG20 alginate is optimum to get "good" quality of hepatocyte microbeads.

In this chapter experiments were carried out in order not only learn the technique but also to optimise the encapsulator settings to produce "good" quality microbeads for cryopreservation. **Figure (4.8)** shows hepatocyte microbeads after production. The microbeads were intact and of regular in shape, and ≤500µm size.

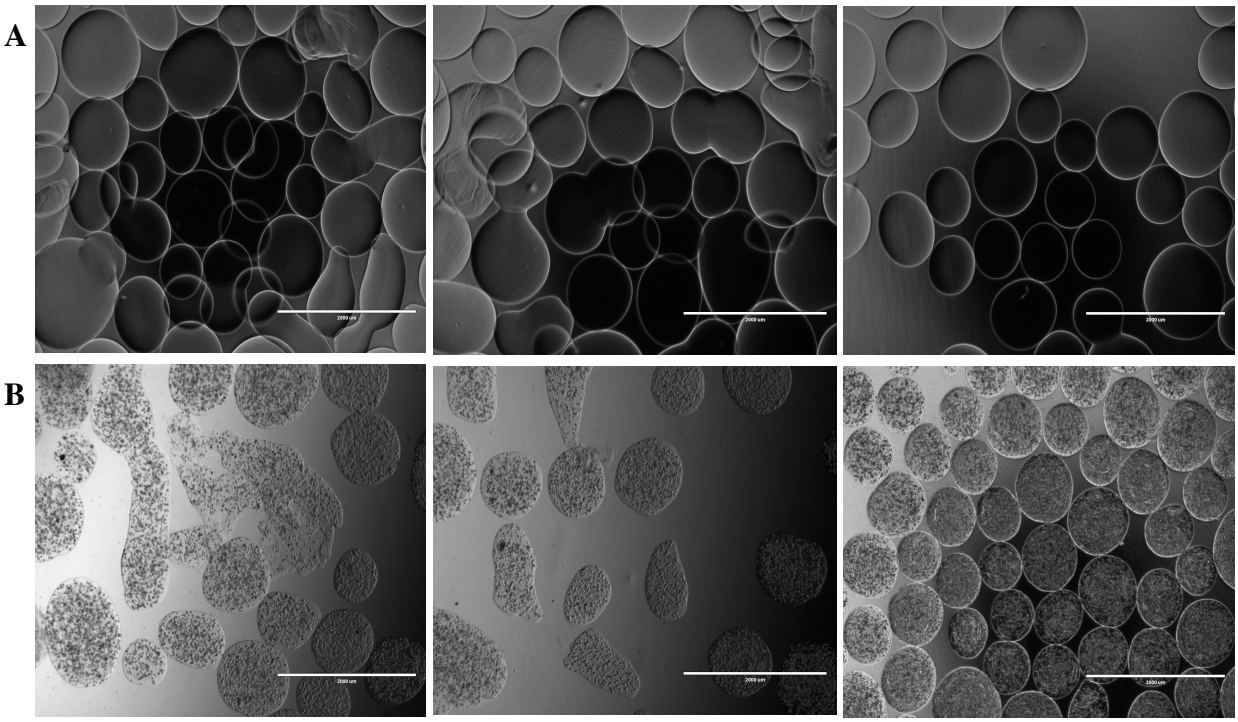


Figure 4.5: Representative images of microbeads to show shape/morphology during optimization using 250µm nozzle **(A)** empty microbeads, **(B)** hepatocyte microbeads.

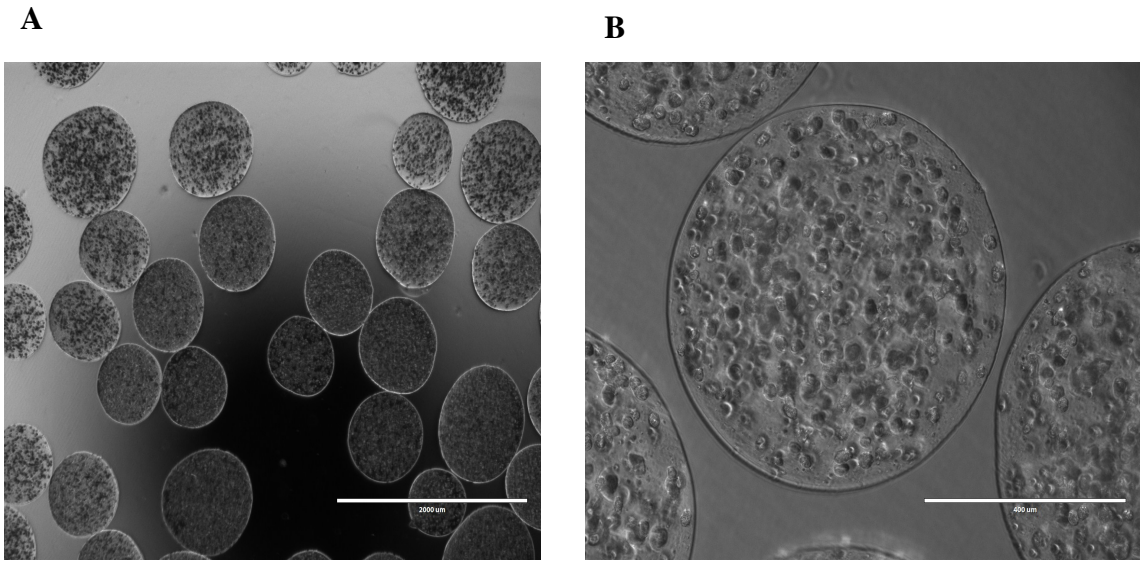


Figure 4.6: A light microscope image showing rat hepatocyte microbeads. [(**A**)magnification =2X; (**B**) magnification =10X].

4.3.5. Assessment of HMBs Activity

Assessment of HMBs function was tested to detect the following: overall activity, Albumin production, urea synthesis and Detoxification. Hepatocyte viabilities were tested using FDA/PI assay. All Assays showed that HMBs were functioning and able to produce hepatic products and function. The activities were tested on day 1,3 and 7, results showed progressive decrease in activity by time **Figure (4.10)**.

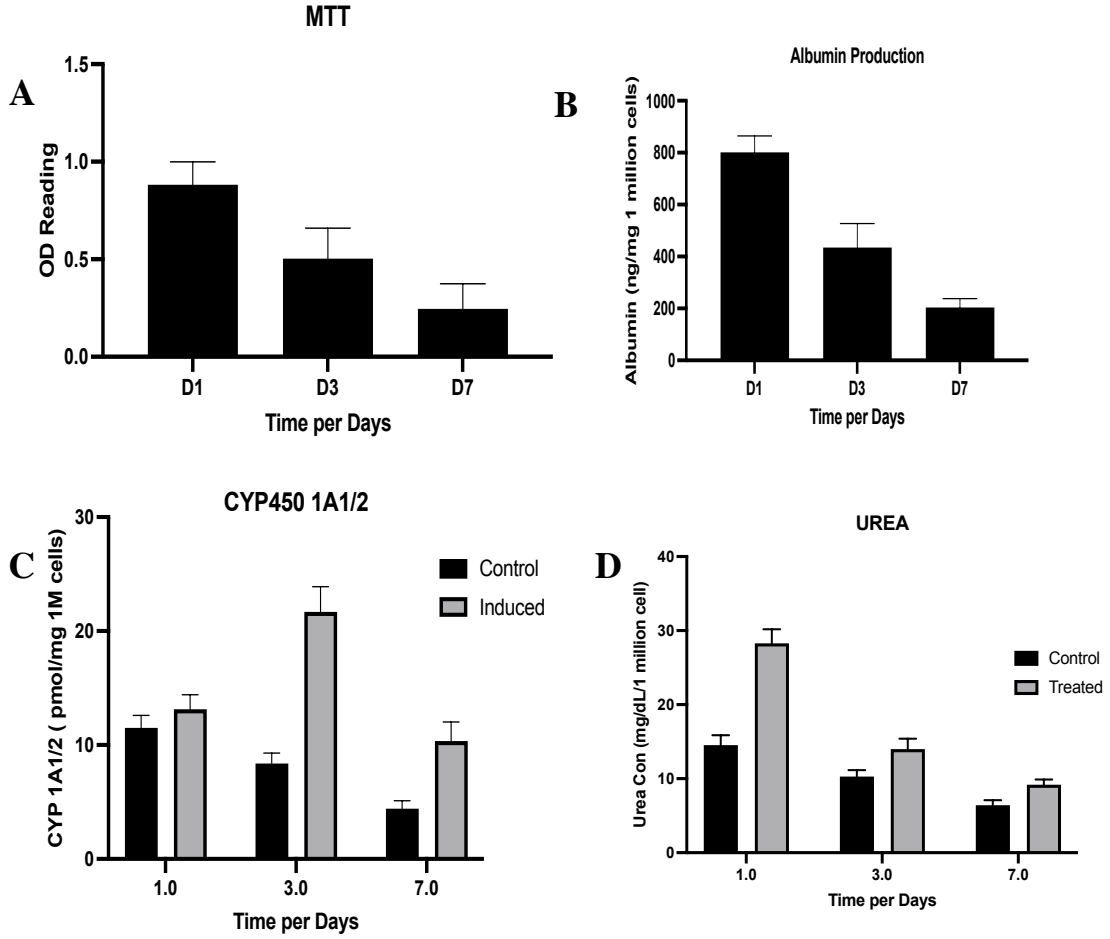


Figure 4.7: Assessment of RMBs activity **A)** Overall activity, **B)** Albumin Production, **C)** CYP450 1A1/2 metabolic activity, D**)** Ammonia Detoxification – Urea Assay (n=11).

4.3.6. Assessment of hepatocyte viability within microbeads

Encapsulated hepatocyte viability was qualitatively assessed using FDA/PI staining to determine whether the cells maintained good viability post encapsulation (**Figure 4.11)**. The images showed percentage of viable cells (green cytoplasm; FDA) and dead cells (red nuclei; PI).

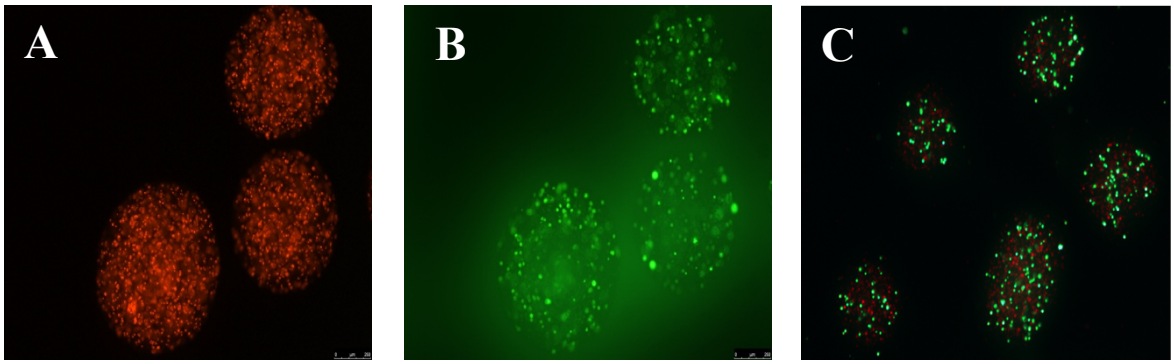


Figure 4.8: Cell viability in alginate-encapsulated hepatocytes assessed by FDA/PI staining. (**A**) Dead cell nuclei stained red (PI) with clear cytoplasm. (**B**) Viable cell cytoplasm stained green (FDA), and (**C**) FDA/PI staining together.

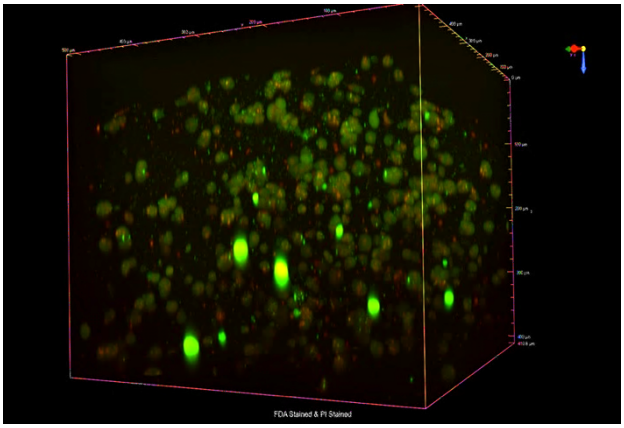


Figure 4.9: 3D imaging of Hepatocyte viability in microbeads using LASER microscopy. A representative photo of FDA (viable; green)/PI (dead; red) stained HMB.

4.4. Discussion and Conclusion

The initial experiments of alginate encapsulations of hepatocytes were conducted by Jitraruch successfully showed that density of $2.5x10^6$ cell per ml of 1.5 % SLG20 alginate is optimum to get good quality of hepatocyte microbeads.

Encapsulator setting for producing optimum size of microbeads with average size between; 500-700 µm were tested and optimized with the following condition: Hepatocyte microbeads were produced using Inotech Encapsulator IE-50R fitted with a 250µm nozzle, using pump speed 300, frequency 1300 Hz and voltages 0.8V resulted with average size of the produced microbeads between 500-700 µm diameter which considered as optimum size of microbeads.

Basic hepatocyte's functions were tested to detect the following: overall activity, Albumin production, urea synthesis and Detoxification. Hepatocyte viabilities were tested using FDA/PI assay. Hepatocyte microbeads were cultured for one week, Microbeads overall activity and liver specific function were tested to find out the viability and activity of the encapsulated hepatocytes within microbeads. All Assays showed that hepatocytes are able to live within microbeads and able to function well.

In vitro data showed that hepatocytes in HMBs were viable throughout the test period (Day 1 till Day 7) and were able to maintain their hepatocyte-specific function and activity such as albumin synthesis, ammonia detoxification (production of urea), and drug metabolism (CYP450 1A1/2). However, there was a progressive decrease in these functions with time.

Chapter 5

Optimization of Hepatocyte Cryopreservation

5.1. Introduction

Cryopreservation of biological samples is global approach, the ability to store samples for future clinical and research purposes was not been able without developing cryopreservation protocols to cryopreserve cells, oocytes, sperms and tissues (Elliott et al., 2017). Cryopreservation at cryogenic temperature can be achieved by different methods such as slow freezing using controlled rate freezer, and vitrification by dropping the samples immediately into liquid nitrogen, In this Chapter hepatocyte cryopreservation is carried out using CRF using different cryopreservation solutions which will be reported in the following section.

5.1.1. Cryopreservation Solution

Several solutions have been developed to cryopreserve cells, sperms, oocytes, tissues and organisms, the main goal of this solutions is to protect the cells during freezing process, where ice formation is the main risk, and can lead to cell death. Ice formation during cryopreservation depends on many factors, including the component of the cryopreservation solution, cooling rate, and temperature (Buhl et al., 2012). To achieve good cryopreservation results cryopreservation solution should contain different component such as solutes, cryoprotectants, anti-freezing agents, pan-caspase inhibitors, or ice blockers (Leibo & Pool, 2011).

During cryopreservation water molecules start forming ice particles below the freezing point which is fatal to cells (Jang et al., 2017). However, with the presence of the solutes in the isotonic solution maintain the osmotic pressure and reduce the chance of ice formation

inside the cells. On the other hand , high concentration of solutes could cause cell dehydration and consequently cell death, therefore choosing optimum solutes concentration is very critical for the cryopreservation process (Bhattacharya & Prajapati, 2016a).

5.1.2. Cryoprotectant Agents

Cryoprotectants or cryo-preservatives are chemicals that are added to protect tissues or cells from freezing induced damages (Bhattacharya & Prajapati, 2016b). Previous studies showed that the addition of some types of cryoprotectants could reduce the freezing induced damage.

Cryoprotectant agents (CPAs) can also be defined as chemicals or compound that dissolve in water and helps lowering the melting point of ice (Devismita & Kumar, 2015). Cryoprotectants are divided into two categories depending on their ability to penetrates cell membrane ***(i) intracellular*** or permeating cryoprotectants such as; ethylene glycol, propylene Glycol, Glycerol and Dimethyl Sulfoxide, this type of cryoprotectant agent (CPA) mainly prevent cell shrinking by reducing the electrolytes concentration inside and outside the cell, the other type is ***(ii) extracellular*** or non-permitting cryoprotectants (e.g. sugars like glucose and sucrose and polymers like milk, dextran's and proteins),This type of CPA enhanced the cell water loss and prevent cell shrinking (Devismita & Kumar, 2015). CPAs are also classified into five types depending on the chemical characteristics: Alcohols and derivatives, Sugars and sugar alcohols, polymers, sulfoxides and amides and amines. Example of alcohol and derivatives are: methanol, ethanol, glycerol, propylene glycol and ethylene glycol. Whereas sugars and sugar alcohols are Glucose, Galactose, Lactose, Trehalose, Raffinose, Mannitol, Sorbitol. On the other hands Polymers could be as; Polyethylene glycol (PEG), Polyvinyl pyrrolidone (PVP), Dextrans, Ficoll, Hydroxyethyl Starch (HES), Serum proteins, milk proteins and Peptones, Nevertheless Sulfoxides and Amides are another type of cryoprotectants

have been used very frequently such as Dimethyl sulfoxide (DMSO), Acetamide, Formamide and Dimethyl acetamide. Since the discovery of DMSO it have been used in many types of cell cryopreservation solution (Awan et al., 2020) In addition to the Amiens; Proline, Glutamine and Betaine are also considered as good Cryoprotectants (Elliott et al., 2017).

Cryoprotectants play an important role in reducing cell damage at low temperature where ice formation can happen and causes cell fraction and nucleation, however selection of cryoprotectants should be done very carefully, and added to the solution in molar concentration, because the excess amount of CPAs could be lethal to the cells, therefore could cause apoptosis which is unfavorable for cryopreservation goals (Bhattacharya & Prajapati, 2016b). When using the slow freezing methods low concentration of cryoprotectants are needed less than 2 M, while high CPAs concentration (4-8 M) is required for the vitrification method (Zheng et al., 2018).

5.1.3. Hepatocyte Cryopreservation

The demand for cryopreserved hepatocyte increased for both clinical and research applications, therefore hepatocyte cryopreservation is the option for long term storage of hepatocyte, for various future uses (Saliem et al., 2012). Hepatocyte cryopreservation is important for the purpose of hepatocyte transplantation as well as the bioartificial liver support system (BAL). In addition, cryopreservation of hepatocyte is a goal to start Bio-Bank of stored hepatocyte or hepatocyte microbeads for future clinical emergency use. However, cryopreservation induced damaged due to ice formation is the main obstacle facing scientists when developing cryopreservation protocols (Wu et al., 2007).

Optimized cryopreservation protocols are required to maintain hepatocyte viability and activity post thawing. In 1980, Fuller and colleagues developed the first successful hepatocyte

cryopreservation protocol, and they were able to recover cryopreserved rat hepatocyte, which was cryopreserved at -196ºC (Fuller et al., 1980).

Addition of cryoprotectants, anti-freezing agents, ice nucleating agents, pan-caspase inhibitors or ice blockers could enhance the cryopreservation mechanism and enhance the retrieval of viable cells and prevent freezing induced damages (Jitraruch et al., 2017).

Several protocols have been developed using different freezing methods with different cryopreservation solutions. The goal is to maintain the viability and function of the hepatocyte post cryopreservation. Which require the development of optimum cryopreservation solution and methods to protect the hepatocytes during the cryopreservation process.

Developing methods for cryopreservation requires understanding the physical and mechanical changes that might occur during cryopreservation, ***Cryopreservation induced damage*** is the biggest challenge facing researchers while developing an optimized protocol for cells or tissues cryopreservation. Cryopreservation induced damage could occur due to ice formation at low temperature. Therefore in order to reduce ice formation during cryopreservation process, cryoprotectants (CPAs) could be added, CPAs are chemicals or compound that dissolve in water and helps lowering the melting point of ice (Devismita & Kumar, 2015). Different studies showed that the addition of the CPAs could enhance the cryopreservation and result in better cell viability and function post thawing (Terry et al., 2006). In addition to cryoprotectants, anti-freezing agents, ice nucleating agents, pan-caspase inhibitors or ice blockers could enhance the cryopreservation mechanism and increases cells retrieval post cryopreservation (Hedayati et al., 2020). Moreover, it was found that, addition of cytoprotectants could reduce the induced molecular cell death pathway, Apoptosis; can be initiated in different stages of cryopreservation, followed by proteolytic caspase cascade pathway(caspase8,9,3), Therefore addition of pan-caspases inhibitor such as

benzyloxycarbonyl-Val- Ala- DL-Asp-fluoromethylketone (Z-VAD) showed improvement of hepatocyte function and viability post thawing (Jitraruch et al., 2017).

In this chapter first ; seven different commercially available cryopreservation solution have been tested for hepatocyte cryopreservation; ***TC-Protector*** (Bio-Rad Laboratories Inc, CA, USA), is ready made cell freezing medium, serum free and contain 10% DMSO, have been tested on different types of cell but not hepatocyte.***CP1*** (Koyoto Pharmaceutical Industry, Koyoto Japan), is cryopreservation medium made especially for the cryopreservation of the hemopoietic stem cells, it contains 10% DMSO, 12% of Hydroxyethyl Starch (HES) and 20% human serum Albumin. Cryopreservation of stem cells from bone marrow, peripheral blood and cord blood cells were tested on this solution for long term cryopreservation. ***pZever*** (Sig; Sigma-Aldrich Ltd.); is cryopreservation medium that does not contain DMSO, no serum neither any animal protein, it contains human protein, and have been tested on Mast cells, dendritic and MSCs, ready to use without any required addition. ***Cryo-JIN*** (REVIVE ORGANTECH Inc, CA, USA); is serum free cryopreservation media, contains anti apoptosis and necrosis compounds, it's made to improve post thawing viability of cells. It is recommended for the cryopreservation of human hepatocyte, neuronal cells, islets cells iPS cells and tissue samples. It contain inhibition of apoptosis and necrosis, as well as inhibitors to minimize freezing stress. ***Cell Banker1;*** is serum containing cryopreservation medium, it have been tested on many cells and cell lines including HepG2 (Nippon Zenyaku Kogyo Co., Ltd, Japan). ***NutriFreez^{TM}D10;*** is ready made freezing media; Animal component free, serum free and protein free, it contains Methylcellulose and 10%DMSO, it has been tested on cryopreserving several types of cells including; Human Mesenchymal cells derived from, Bone Marrow, Adipose Tissue, Umbilical Cord Tissue and Dental Pulp tissue, Human Embryonic Stem cells (hESCs) and Induced Pluripotent Stem Cells (iPSC), and many mammalian cell lines such as; HepG2, MRC-5, HeLa and many other cell lines. It does not contain any growth

factors neither serum nor proteins. Finally, ***University of Wisconsin solution;*** (UW; Bristol-Myers Squibb AB, Sweden) is the main solution used for clinical grade hepatocyte cryopreservation at King's College London Hospital supplemented with 5% glucose and 10% DMSO. Also, UW is used as organ preservation solution; the main ingredients of UW as provided by manufactuer: K^+lactobionate (100 mM), KH_2PO_4 (25 mM), Na^+ (30 mM), Raffinose (30 mM), Adenosine (5 mM), Glutathione (3 mM), Hydroxyethyl starch (50 g/L), Allopurinol (1 mM), Dexamethasone (8 mg/L) and Penicillin (200,000 U/L), with osmolarity 310 mOsM and pH 7.4. Although UW contains raffinose, glucose addition could further enhance its cold-storage effect(s) and may act as a cryoprotectant.

The aim of this work is to test commercially available cryopreservation solutions supplemented with/without cryoprotectant agent(s) for cryopreservation of hepatocytes and compare them to UW with 5% glucose and 10% DMSO solution.

5.2. Materials and Methods

5.2.1. Materials

5.2.1.1. Cryopreservation Solutions and Additives

Seven different commercially available cryopreservation solutions were used: ***Cell Banker 1*** (Nippon Zenyaku Kogyo Co., Ltd, Japan). ***Nutri Freez***^{TM} D10 (Sartorius, BI,USA), ***TC-Protector*** (Bio-Rad Laboratories Inc, USA), ***pZever*** (Sig; Sigma-Aldrich Ltd., USA), ***CP1*** (Koyoto Pharmaceutical Industry, Koyoto Japan), ***Cryo-JIN*** (REVIVE ORGANTECH Inc, CA, USA) and ***University of Wisconsin solution*** (***UW;*** Bristol-Myers Squibb AB, Sweden). Other condition were tested using UW solution with other additive such as; ***Glucose*** 50% (Hameln pharmaceuticals ltd, UK), ***Dimethyl sulfoxide*** (DMSO; Sigma-Aldrich Ltd., USA), ***Trehalose*** (Sigma-Aldrich Ltd., USA), ***Z-VAD*** (Promega., UK), ***Ethylene Glycol*** (Alfa Aesar, thermo fisher scientific, UK) and Polyvinylpyrrolidone ***PVP*** (Sigma-Aldrich Ltd., USA).

5.2.1.2. Chemicals and Solutions for hepatocyte Assays

All chemicals and assays to test hepatocyte-specific activity used were as described in Chapter 2, Section (2.2.4) till Section (2.2.8) respectively.

5.2.2. Methods

5.2.2.1. Experimental design

In this chapter seven different cryopreservation solution have been tested for their efficacy to cryopreserve hepatocytes, each solution contains different types of cryoprotectants and additives **TC-Protector, Cell Banker1, CP1, Cryo-JIN, NutriFreezTMD10, pZever** and **UW solution Table (5.1)**. All solutions were tested and compared with UW solution with 10% DMSO and 5% glucose, Then UW solution was tested with different additives cryoprotectant to test its efficacy for hepatocyte cryopreservation, 1.5 M ETG, 0.2M Trehalose, 60µM Z-VAD, and 2% PVP.

Table 5.1: Cryopreservation Solutions [Commercially Available]

Cryopreservation Solution	*Cryoprotectant*	*company*
1- **TC-Protector**	- 10 % DMSO	Bio-Rad Laboratories Inc, CA, USA
2- **Cell Banker1**	- Serum	Nippon Zenyaku Kogyo Co., Ltd, Japan
3- **CP1**	- 10% DMSO - 12% HES	Koyoto Pharmaceutical Industry, Koyoto Japan
4- **Cryo-JIN**	- N/A	REVIVE ORGANTECH Inc, CA, USA
5- **NutriFreez^{TM}D10**	- 10% DMSO - Serum free. - methylcellulose	BI ;Biological Industries
6- **pZever**	- Serum Free	Sig; Sigma-Aldrich Ltd
7- **UW**	- 10% DMSO - 5% Glucose	UW; Bristol-Myers Squibb AB, Sweden

After testing the seven commercially available hepatocyte cryopreservation solution and comparing them with UW solution, more optimisation conditions were tested on UW solution with different cryoprotectants concentration based on earlier publication as listed in **Table (5.2)**.

Table 5.2: Concentration of Cryoprotectants added to UW solution		
Cryoprotectant	***Concentration***	***Reference***
DMSO	10%	Jitraruch et al., 2017
Trehalose	0.2M	Katenz et al., 2007
Z-VAD	60µM	Jitraruch et al., 2017
PVP	2%	Illouz et al., 2008
ETG	1.5 M	Arutyunyan et al., 2018

Eight different conditions using UW solution with different types and concentrations of cryoprotectants have been tested for hepatocyte cryopreservation as shown in **Table (5.3)**, each condition was compared against **Condition Number 1 (Con 1)** which is mainly used at KCH as hepatocyte cryopreservation solution and consist of UW with 10% DMSO and 5% Glucose. **Con 1**, was used considered as control protocol.

Table 5.3: UW solution with different concentration of cryoprotectants			
Cond 1	**Cond 2**	**Cond 3**	**Cond 4**
- UW - 10% DMSD - 5% glucose	- UW - 10% DMSD - 5% glucose - 60 µM Z-VAD	- UW - 10% DMSD - 5% glucose - 60 µM Z-VAD - 2% PVP	- UW - 10% DMSD - 5% glucose - 60 µM Z-VAD - 1.5 M ETG
Cond 5	**Cond 6**	**Cond 7**	**Cond 8**
- UW - 10% DMSD - 5% glucose - 60 µM Z-VAD - 2% PVP - 1.5 M ETG	- UW - 10% DMSD - 0.2 M Trehalose	- UW - 10% DMSD - 0.2 M Trehalose - 60 µM Z-VAD	- UW - 10% DMSD - 0.2M Trehalose - 60 µM Z-VAD - 2% PVP - 1.5 M ETG

5.2.2.2. Hepatocyte Cryopreservation

Cryopreservation processes were carried out using **Controlled-Rate Freezer** (CRF; model: Kryo10 from Planer, UK). 5 ml cryopreservation tubes were used, 1 ml of 1 $X10^7$ cells were added in each tube and completed with 4 ml of the cryopreservation solution. All cells were cryopreserved using controlled rate freezer, protocol program described in **Table (5.4)** then sample ware stored in -140 ºC freezer until thawing. Slow cooling rates were used to allow cells to cool down gradually and allowing cryoprotectants to take their action(s). Once cells are cooled down, a shock cooling was introduced to cells to reduce ice nucleation. In this protocol cells were cooled down quickly to -28ºC (Table 5.4, step 4).

Table 5.4: Controlled-Rate Freezer Program for hepatocyte cryopreservation

Step	Start Temperature	Rate	Time	End Temperature
1	8ºC	1ºC/min	8 min	0ºC
2	0ºC	Hold	8 min	0ºC
3	0ºC	-2ºC/min	4 min	8ºC
4	-8ºC	-35ºC/min	33 Sec	-28ºC
5	-28ºC	-2.5 ºC/min	2 min	-33ºC
6	-33ºC	+2.5 ºC/min	2 min	-28ºC
7	-28ºC	-1 ºC/min	32 min	-60 ºC
8	-60 ºC	-10 ºC/min	4 min	-100 ºC
9	-100ºC	-20 ºC/min	2 min	-140 ºC

5.2.2.3. Hepatocyte Thawing and Culturing

Two-week post-cryopreservation cells were quickly thawed (2-3min) in a 37°C water-bath with gentle shaking.

Thawed cell suspension was diluted with ice-cold EMEM, and centrifuged at 50Xg, 4°C for 5 min. Hepatocyte was checked for cell number and viability using trypan blue and light microscope; followed by plating. Hepatocytes were plated in collagen-coated plates, at 50,000 cells/well in 200µl WEM (96-well plates for MTT and SRB assays), and 250,000 cells/well in 500µl WEM (24-well plates for albumin, urea, and CYP P450 assays). Medium was changed as appropriate according to the time point 24, 48 & 72h.

5.2.2.4. Hepatocyte Activity Assays

Hepatocyte Overall Metabolic Activity were conducted as described in Chapter 2 (MTT Assay; Sections: 2.2.4), Cell Attachment using Sulphorhodamine B (SRB Assay; Sections: 2.2.5), Albumin (ELISA Assay; Sections: 2.2.6), Ammonia detoxification Urea Assay; Sections :2.2.7), Cytochrome P450 EROD Assay (CYP 450 A1/2; Sections: 2.2.8).

5.2.2.5. Total Cell Lysat and Protein Content Quantification

Protein content quantification was carried out by using Bio-Rad DC™ Protein Assay kit as described in Chapter 2, Sections (2.2.9) & (2.2.10).

5.3. Results

In this chapter human and rat hepatocyte were cryopreserved with 7 different ready-made cryopreservation solution and tested for its efficacy for hepatocyte cryopreservation, then UW solution was tested with different cryoprotectant additions and tested for its efficiency for hepatocyte cryopreservation, obtained results are presented in this chapter.

5.3.1. Comparison of Readymade Cryopreservation Solutions to UW Solution

The first part of this chapter covers the initial experiments of hepatocytes cryopreservation by testing/comparing seven different ready-made commercially available cryopreservation solutions. Human and rat hepatocytes were cryopreserved using slow freezing method by using controlled rate freezer. Cryopreserved hepatocytes were moved to -140 ºC deep freezer for one to two weeks, then hepatocytes were thawed and tested for its viability and activity.

5.3.2. Human Hepatocyte Cryopreservation

The first part of the experiment was conducted on human hepatocyte n=8, fresh and cryopreserved hepatocyte were tested and assessed for function and viability as listed in bellow sections.

5.3.2.1. Human Hepatocytes Viability Determination / trypan blue exclusion post cryopreservation

Fresh and cryopreserved hepatocyte cell viability were determined using trypan blue exclusion. Although each solution gave different cell viability, there were significant differences between the tested solutions TC-Protector, CP1 and pZever solutions showed significant low viability comparing with other solution. however, Cryo-JIN, Cell Banker and NutriFreez solutions gave similar cell viability post-thawing compared to UW solution.

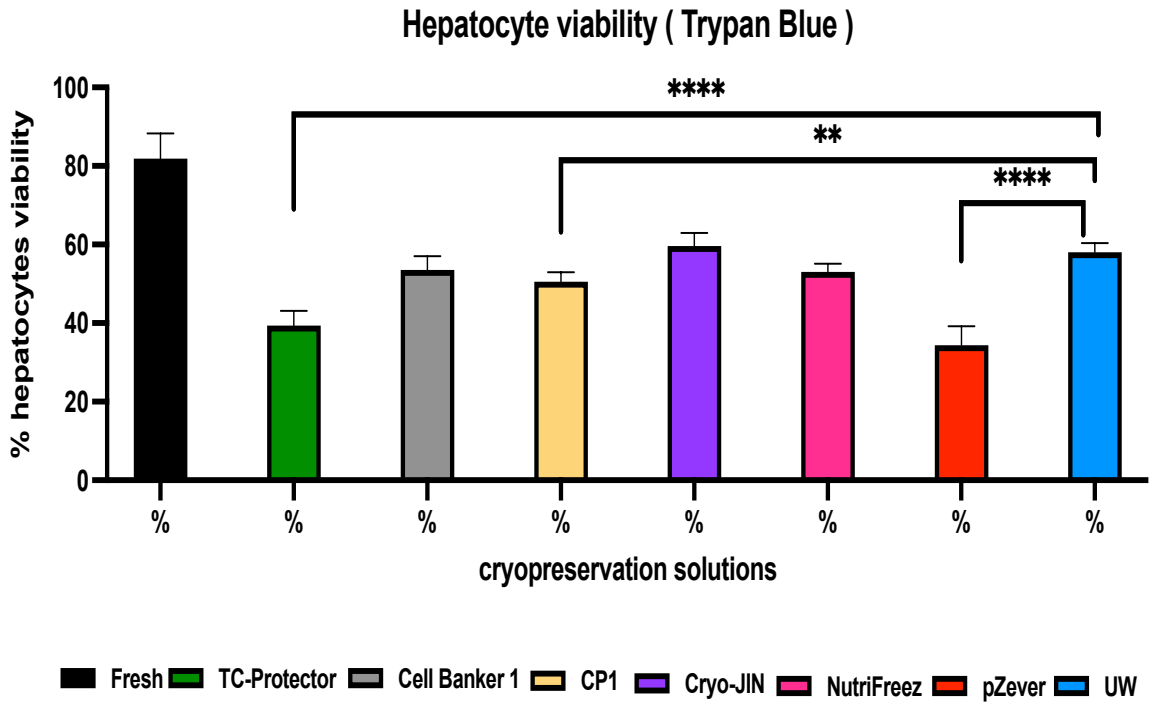


Figure 5.1: Human hepatocyte viability after two weeks of cryopreservation using seven readymade cryopreservation solutions, n=8, Cryo-JIN , Cell Banker and NutriFreez solutions gave similar cell viability post-thawing compared to UW solution. TC-Protector, CP1 and pZever solutions showed significant low viability comparing with other solution [**P:= 0.0023, ****P : <0.0001; UW = University of Wisconsin].

5.3.2.2. Human Hepatocyte Overall Metabolic Activity- MTT Assay Post Cryopreservation

The overall metabolic activity of the fresh and cryopreserved hepatocyte tested at 24, 48 and 72 hours **(Figure 5.3)**, hepatocytes cryopreserved in UW, NutriFreez, and Cell Banker1 showed similar activity levels, however hepatocytes cryopreserved with TC-Protector, CP,1 and pZever solutions showed significant low activity compared to other solution. On the other hand, hepatocytes cryopreserved with Cryo-JIN showed a significantly higher baseline overall activity compared to other cryopreservation solutions (P<0.0001).

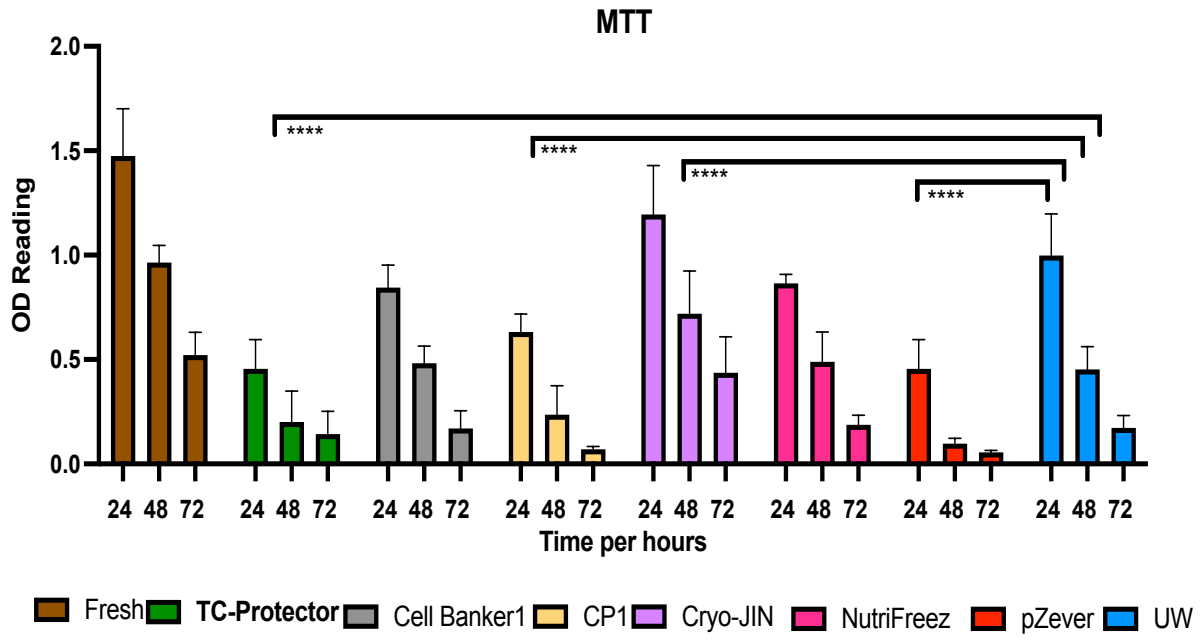


Figure 5.2: MTT results for human hepatocyte fresh and cryopreserved. The data are presented as mean ± SD, n=8. Two-way ANOVA was used to compare the different solutions, and multiple comparisons using UW as a control. The results shows that cells activity reduced with time and most of the solutions, TC-Protector and pZever solutions showed significant low activity comparing with other solution. However, all cryopreserved hepatocytes showed a significant drop in overall activity, Cryo-JIN, NutriFreez and Cell Banker 1 maintained a higher activity compared to other solutions. [****:<0.0001; UW = University of Wisconsin].

5.3.2.3. Human Hepatocyte Attachment - Sulphorhodamine B (SRB) Assay Post Cryopreservation

Cell attachment of fresh and cryopreserved hepatocyte tested at 24, 48 and 72 hours, **Figure (5.4);** results of thawed and cultured cryopreserved hepatocytes showed that there was a slight decrease in hepatocytes attachment level of with time points. However, cells cryopreserved with Cryo-JIN showed significantly higher level of attachment compared to UW at baseline (P= 0.0100); were Cell Banker1, NutriFreez and CP1 gave similar result of UW, on the other hand significant drop-in activity resulted when cryopreserved with TC-Protector and pZever solutions (P<0.0001).

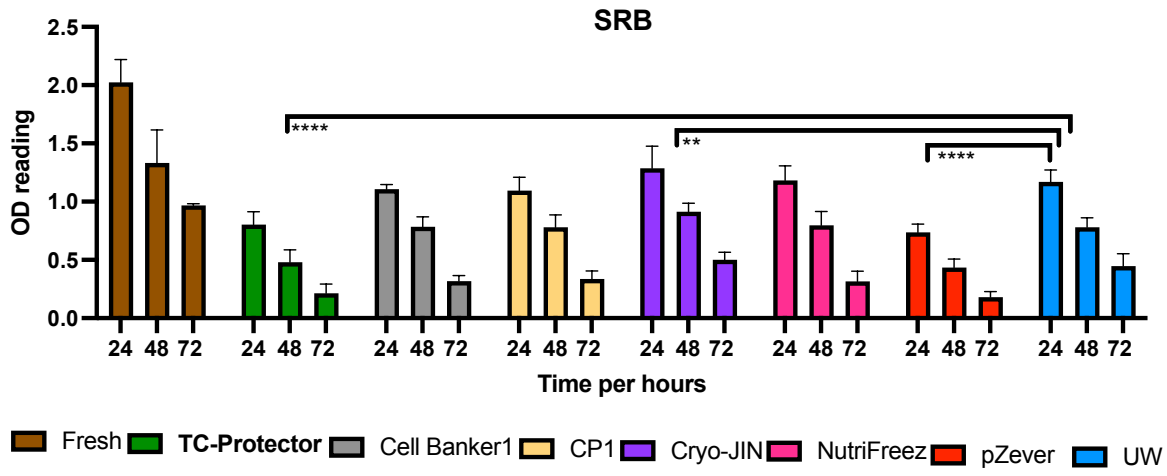


Figure 5.3: SRB results of human hepatocyte cryopreserved for one week. Data presented as mean ± SD. This result refers to n=8. Tow-way ANOVA was used to compare the different solutions, and multiple comparisons using UW as a control. The results show that cells attachment slightly decreased with time especially in cryopreserved cell culture, however Cryo-JIN maintained a significantly higher attachment compared to all other cryopreserved hepatocytes. [**: 0.0100, ****:<0.0001; UW = University of Wisconsin].

5.3.2.4. Human Hepatocyte Ammonia detoxification -Urea Assay Post Cryopreservation

Urea production is another hepatocyte-specific function that is based on detoxification of ammonia. This test was carried out by challenging hepatocytes with ammonium chloride and measuring the urea production level. The data presented in **Figure (5.4)** shows that hepatocytes cryopreserved in Cryo-JIN had the highest urea production level compared to other cryopreservation solutions. Where TC-Protector and pZever showed low level of urea production comparing to the other solutions, CP1, NutriFreez, and Cell Banker1 had a slightly higher level of urea production compared to UW.

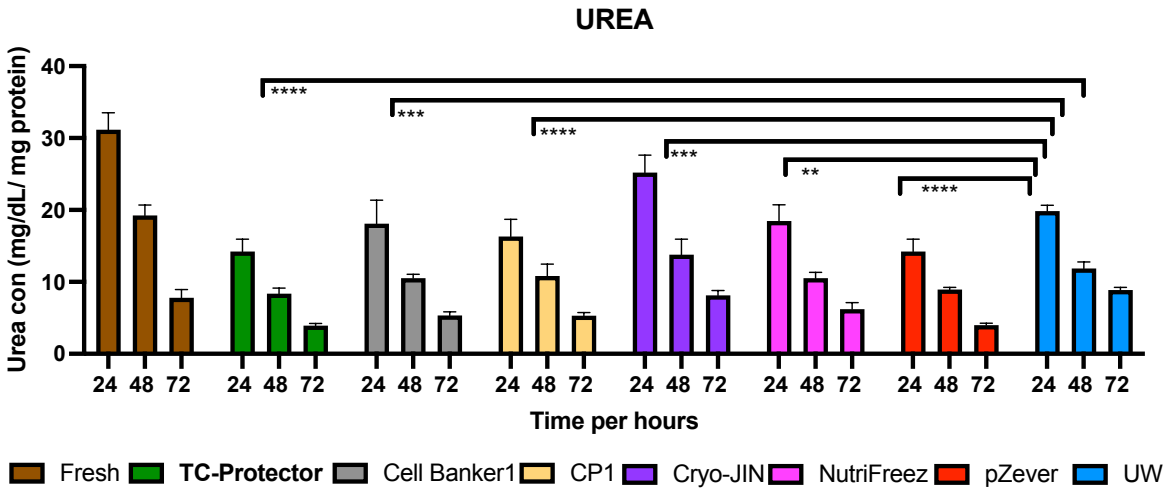


Figure 5.4: Urea production by human hepatocytes in culture. Data shows that urea synthesis was synthesized at statistically significant levels when Cryo-JIN, Cell Banker, NutriFreez and CP1solutions were used comparing with the other solutions. There was a significant drop in urea production when pZever and TC-protector solutions were used [P: ****:< 0.0001, ***: 0.0001, **:0.0023].

5.3.2.5. Human Hepatocyte Cytochrome P450 EROD Assay (CYP 450 A1/2) Post Cryopreservation

Cytochrome P450 (CYP450) activity in the liver is one of the essential functions for drug metabolism and toxins detoxification. Ethoxy resorufin-O-demethylase (EROD) assay is used to determine the level of induction of the xenobiotic-metabolizing enzyme cytochrome P-450 (CYP) 1A1/2 during the detoxification process by hepatocytes. Human hepatocytes were induced by Omeprazole, for 24, 48 and 72 hours, then florescence of resorufin standards and samples were read at excitation of 530 nm/emission of 590 nm using FLUOstar Omega plate reader.

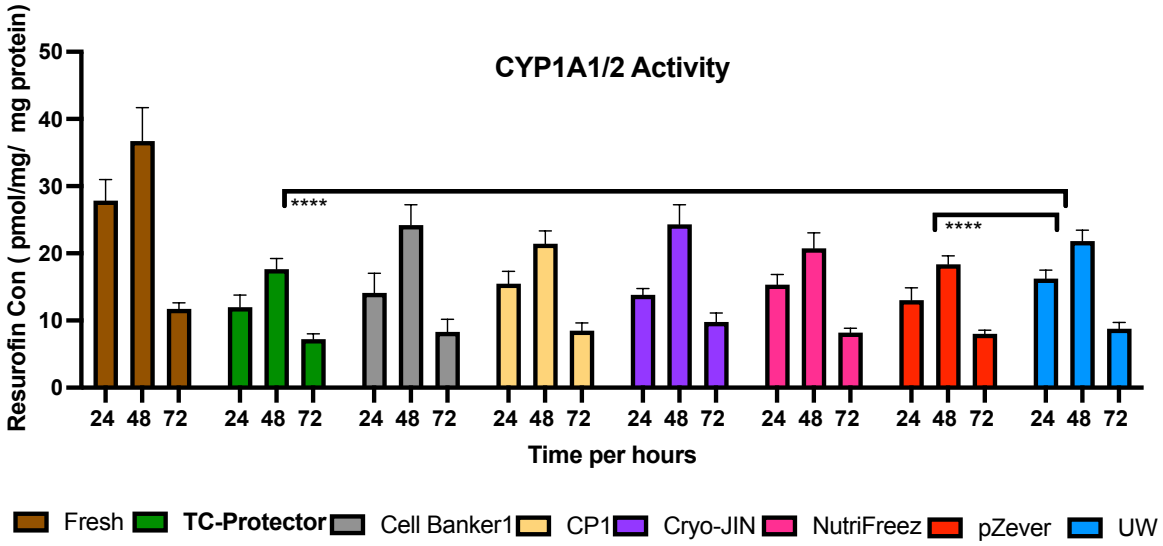


Figure 5.5: EROD assay of cultured human hepatocyte post cryopreservation**.** Results showed that cryopreserved hepatocyte with UW, Ce11 Banker1, CP1 and NutriFreez solutions, were able to detoxify the xenobiotic agent through the activation of CYP 450 metabolic enzymes with the same lever, however the activity of hepatocyte cryopreserved with TC-Protector and pZever were significantly lower than other solution and reduced by time.

5.3.2.6. Human Hepatocyte Albumin Production - ELISA Assay Post Cryopreservation

Albumin is one of the most important proteins in the body, produced by hepatocytes in the liver. Functioning and living hepatocytes are able to synthesise albumin, therefore, to determine the activity of cryopreserved hepatocyte, albumin was measured in different time point.

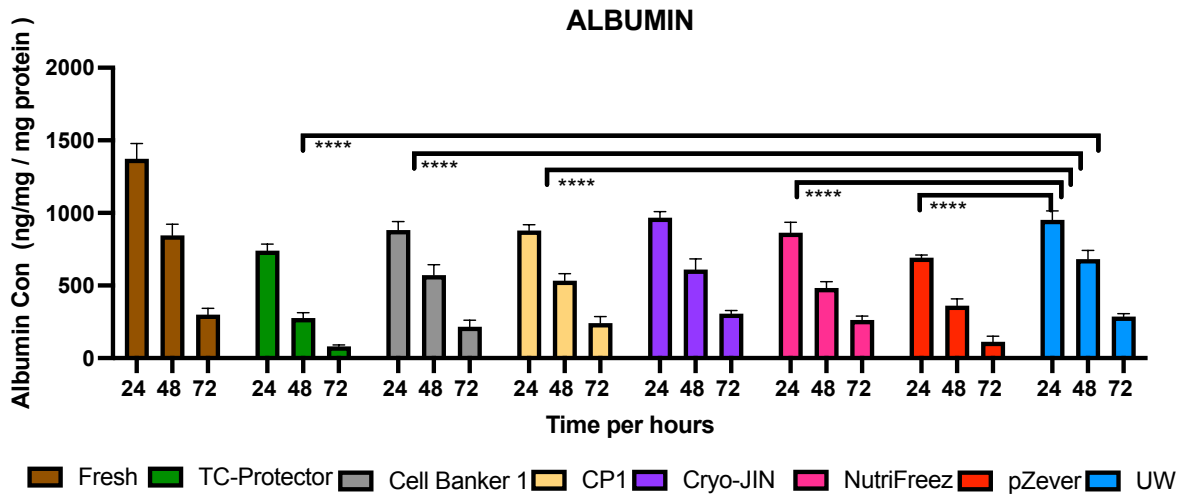


Figure 5.6: Albumin synthesis activity of cultured human hepatocyte post cryopreservation. Results shows that all fresh and cryopreserved hepatocytes were able to produce albumin and reduced by time, however all solutions showed significant lower level of albumin production except Cryo-JIN and UW that maintained higher levels of albumin production comparing with the whole other solutions.

5.3.3. Rat Hepatocyte Cryopreservation Results

The following sets of experiments were repeated using rat hepatocyte (n=7). Rat hepatocytes were cryopreserved using the same seven readymade cryopreservation solutions using CRF, the hepatocytes were thawed and assessed for cell viability, overall activity, and hepatocyte-specific activities one week post cryopreservation for the time point 24, 48 and 72 hours.

5.3.2.1. Overall Metabolic Activity- MTT Assay

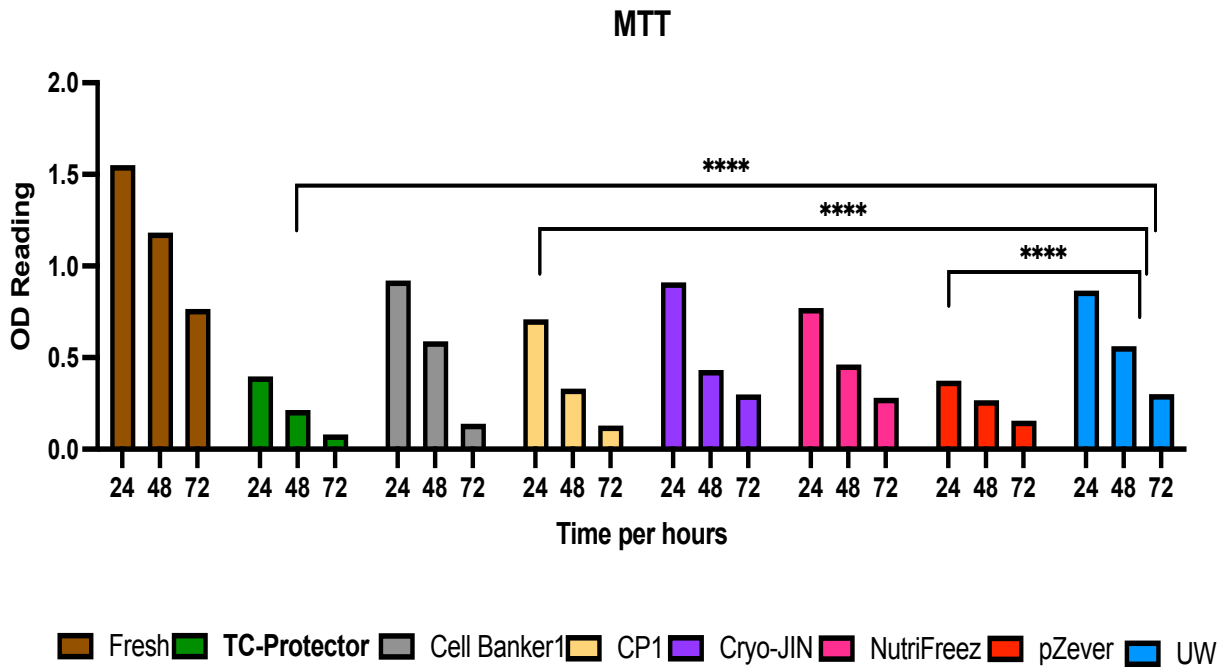


Figure 5.7: Overall activity (MTT) of cryopreserved rat hepatocytes compared to fresh hepatocytes. Data presented as mean ± SD. This data presented (n=7), were analyzed using two-way ANOVA test to compare the different solutions, and multiple comparisons using UW as a control. Results showed that cryopreserved hepatocytes using TC-Protector, CP1 and pZever solutions had significant drop in overall activity with tested time point 24, 48 & 72 hours (P: <0.0001), however UW, Cryo-JIN, NutriFreez and Cell Banker1 maintained higher activity compared to other solutions.

5.3.3.2. Rat Hepatocytes Attachment - Sulphorhodamine B (SRB) Assay Post cryopreservation

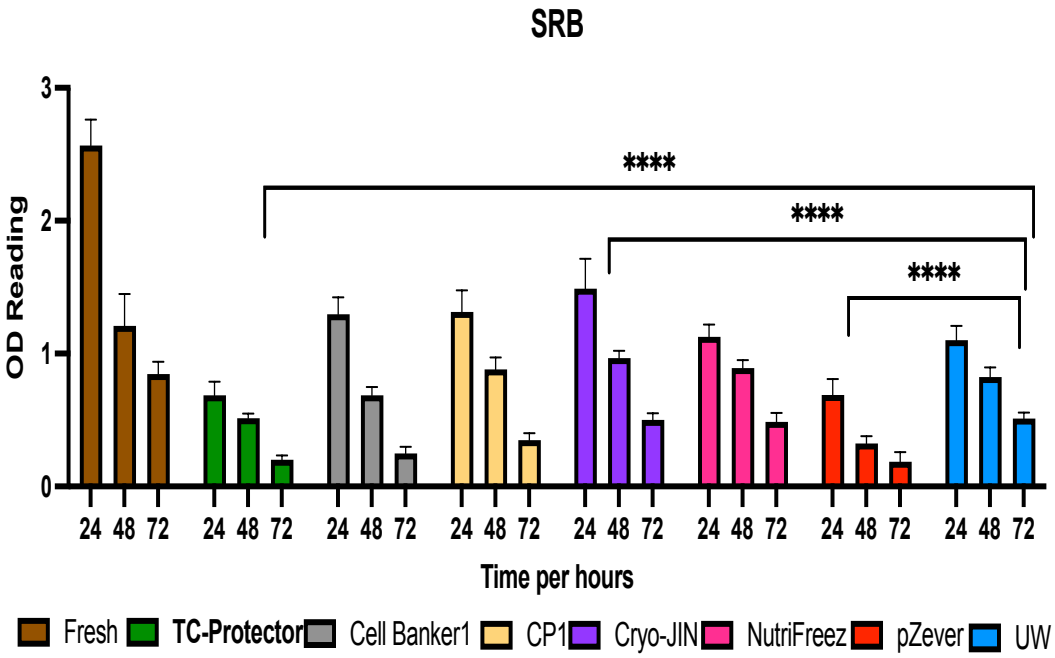


Figure 5.8: Cell attachment (SRB) of cryopreserved rat hepatocytes compared to fresh cells data presented (n=7). The results shows that cells attachment slightly decreased with time especially in cryopreserved hepatocyte with pZever and TC-Protector solutions(P:<0.0001), however Cryo-JIN solution maintained a significantly higher attachment compared to all other cryopreserved hepatocytes. UW, Cell Banker1, CP1 and NutriFreez solution gave similar activity of cell attachment [****:<0.0001; UW = University of Wisconsin].

5.3.3.3. Rat Hepatocytes Ammonia detoxification - Urea Assay Post Cryopreservation

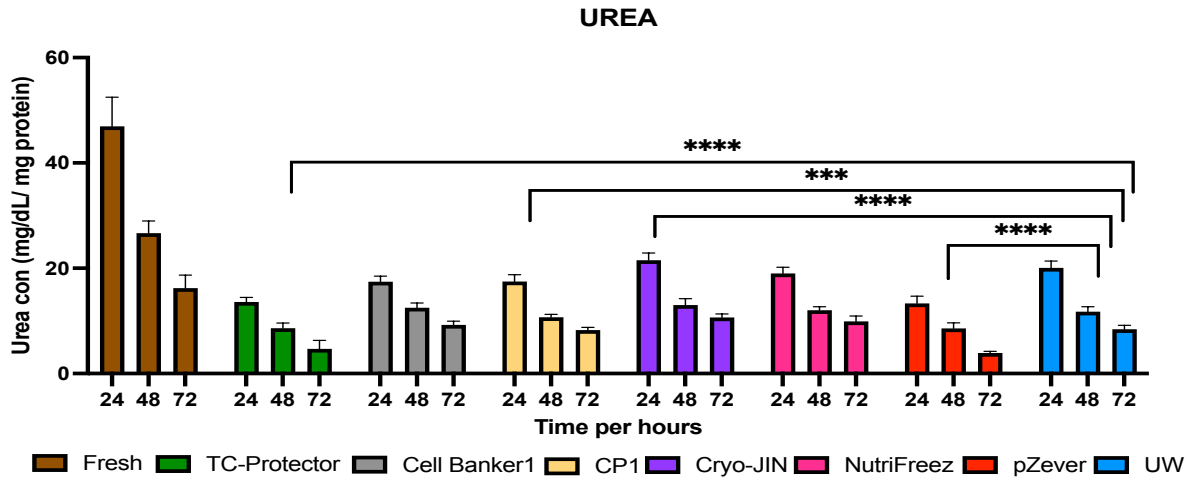


Figure 5.9: Urea synthesis (ammonia detoxification) by cryopreserved rat hepatocytes compared to fresh cells data presented (n=7), at time points 24, 48, and 72 hours. Urea assay showed that Cryo-JIN, had significant increase in urea production, were Cell Banker and, NutriFreez maintained similar level of urea production of UW solution, Nevertheless CP, pZever and TC-Protector solutions showed significant decrease levels of Urea production comparing with other solutions. [****P: < 0.0001, ***P: 0.0006].

5.3.3.4. Rat Hepatocytes Cytochrome P450 EROD Assay (CYP 450 A1/2) Post Cryopreservation

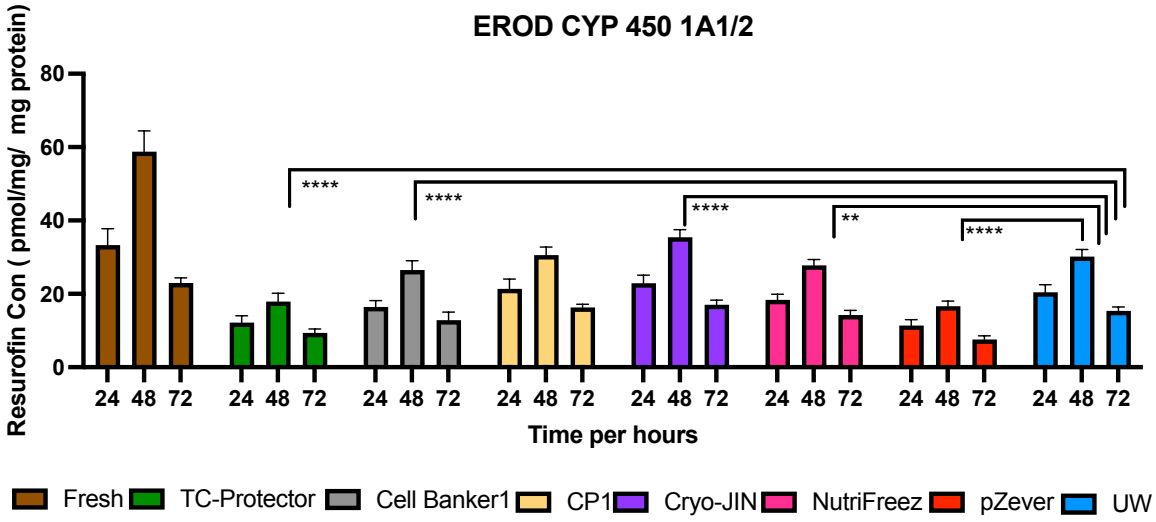


Figure 5.10: Cytochrome P450 (1A1/2) activity in cryopreserved rat hepatocytes compared to fresh cells data presented (n=7). The results shows that all tested solution had significant drop of CYP450 activity except Cryo-JIN solution, which maintained significant higher level of activity post thawing with the different time point. [**P: = 0.0092, **** P: < 0.0001].

5.3.3.5. Rat Hepatocyte Albumin Production - ELISA Assay Post Cryopreservation

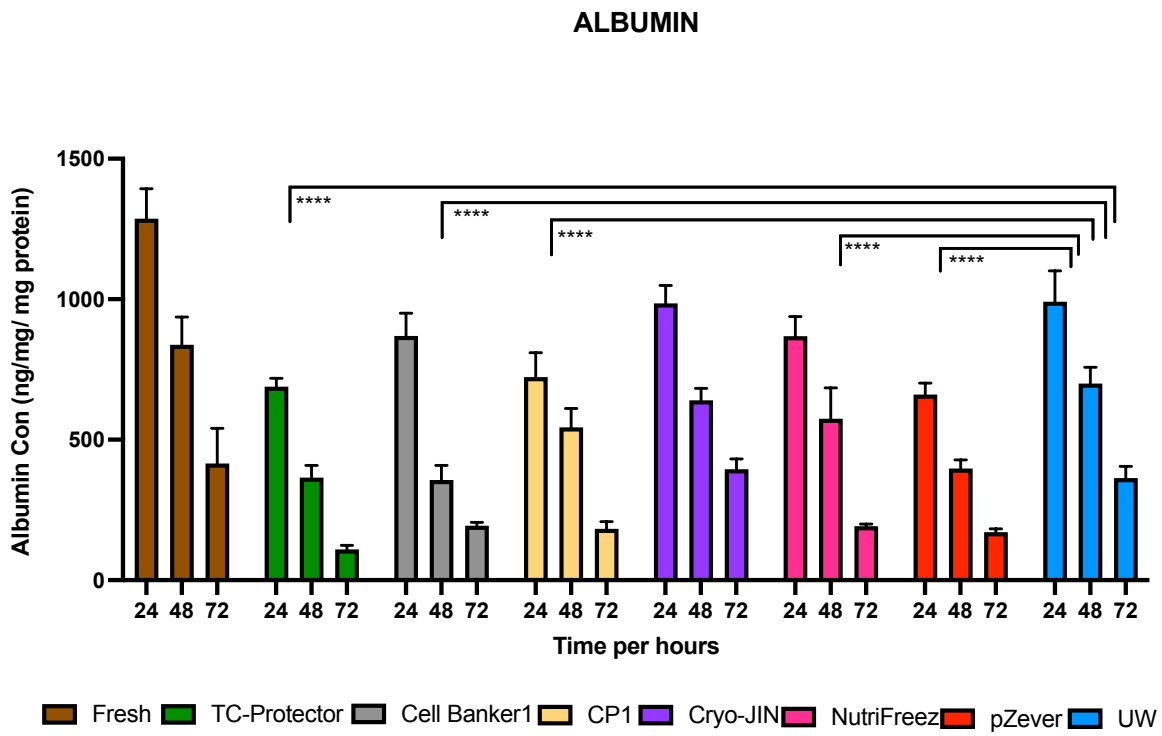
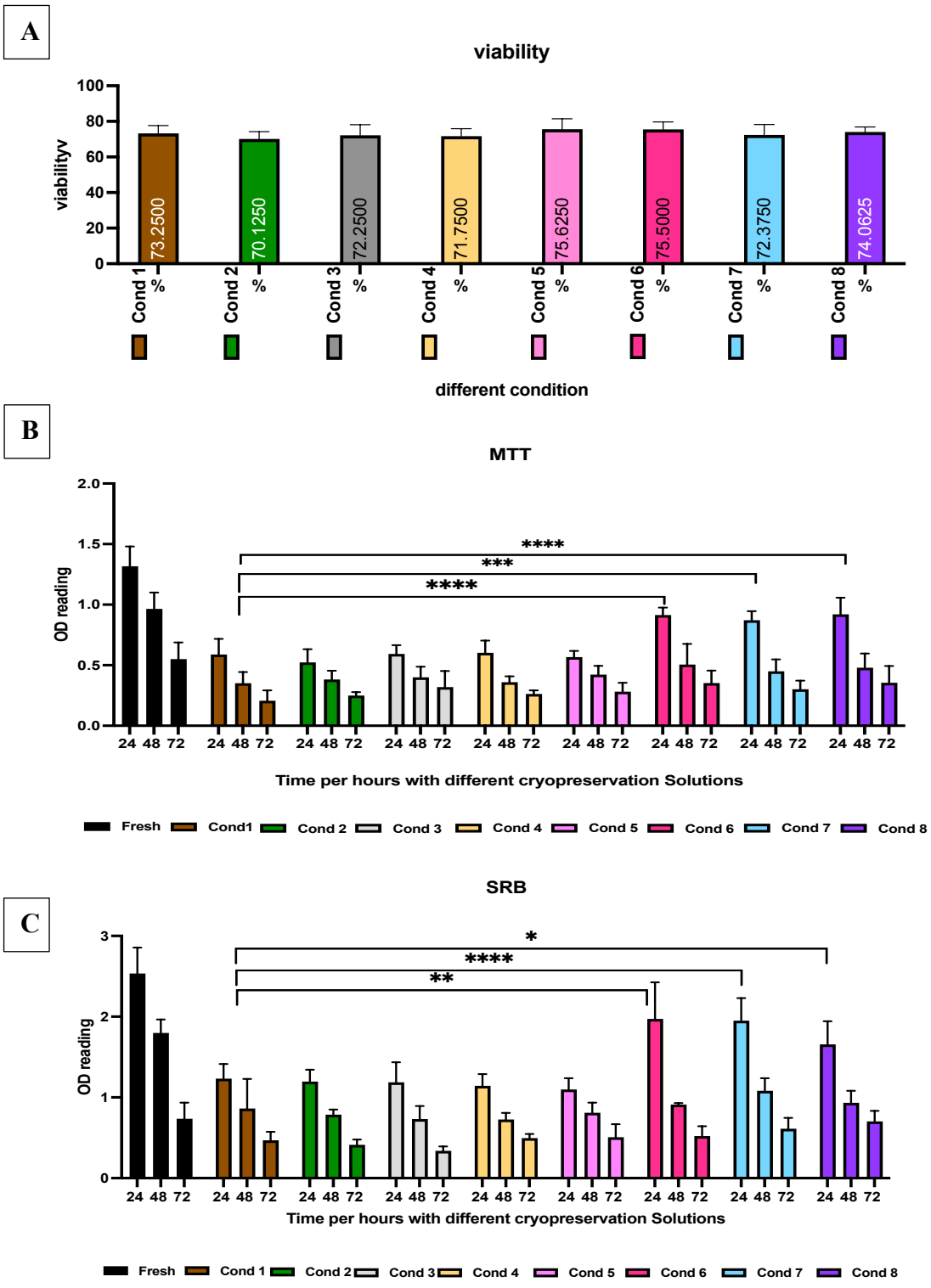


Figure 5.11: Albumin synthesis by cryopreserved rat hepatocytes compared to fresh cells data presented (n=7). The results obtained suggests that all tested solution showed significant drop of albumin production except Cryo-JIN solution maintained almost similar level of albumin production with UW solution post thawing with the different tested time point. [**** P: < 0.0001].

5.3.4. Optimization of New Cryopreservation Condition with UW Solutions

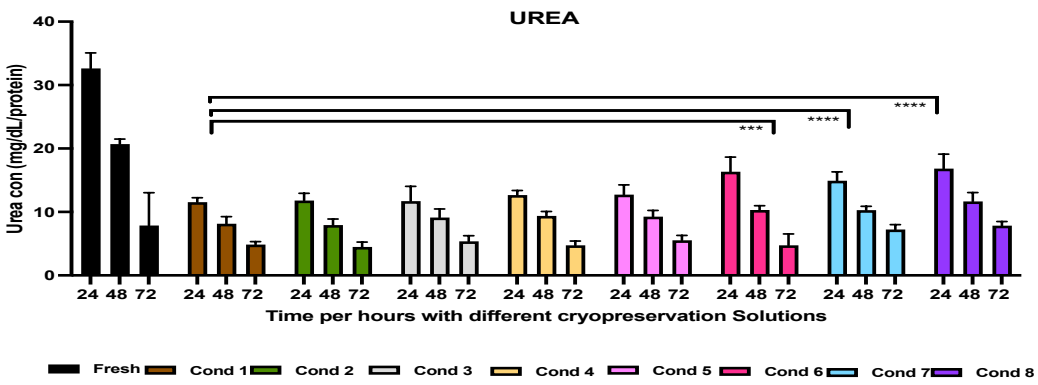
After testing and comparing the 7 different ready-made cryopreservation solutions, UW solution was tested with other cryoprotectants in eight different conditions as listed in **table (5.3)**, The eight different cryopreservation conditions were used to test their efficiency for hepatocyte cryopreservation, each contained different type of cryoprotectants (CPAs) and compared with the condition Number 1 which consist of (UW + 5% glucose + 10%DMSO) which is manly used for hepatocyte cryopreservation at KCH. Each cryoprotectant chosen and added with different concentration based on earlier published cryopreservation studies. Number of experiments were limited to n=8 due to issues with the collagenase activity used for hepatocytes isolation.

One-week post-cryopreservation cells were quickly thawed in a 37°C water-bath with gentle shaking. Thawed cell suspension was diluted with ice-cold EMEM, and centrifuged at 50xg, 4°C for 5 min. The cell pellet was checked for cell number and viability followed by plating for different assays and activity testing.



Cryoprotectant with UW Solution		Cond 1	Cond 2	Cond 3	Cond 4	Cond 5	Cond 6	Cond 7	Cond 8
-	DMSO 10%	+	+	+	+	+	+	+	+
-	Glucose 5%	+	+	+	+	+	-	-	-
-	Z-VAD 60 µM	-	+	+	+	+	-	+	+
-	PVP 2%	-	-	+	+	+	-	-	+
-	ETG 1.5 M	-	-	-	+	+	-	-	+
-	0.2M Trehalose	-	-	-	-	-	+	+	+

D



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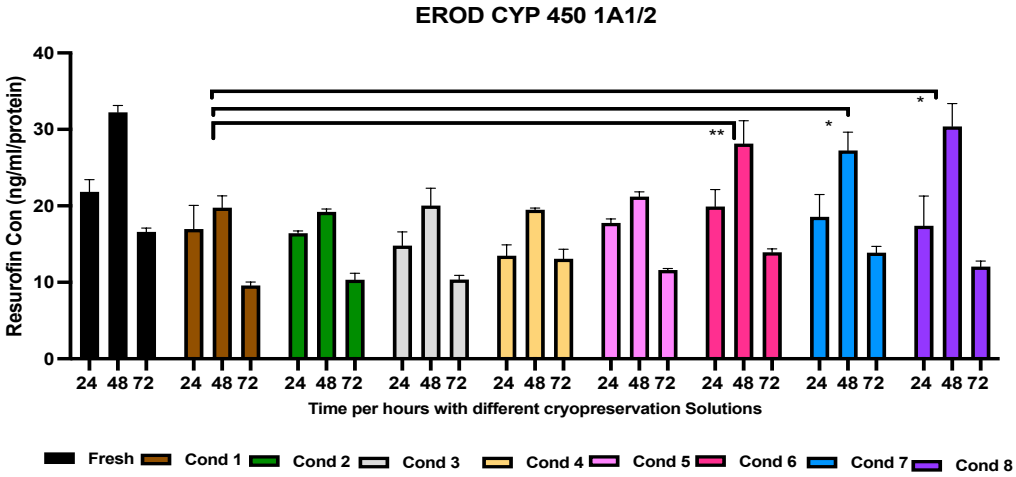

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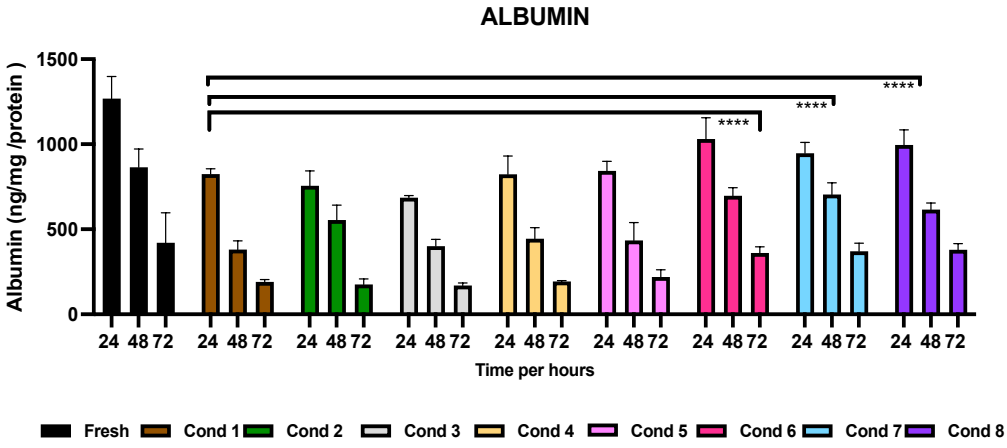


Cryoprotectant with UW Solution		Cond 1	Cond 2	Cond 3	Cond 4	Cond 5	Cond 6	Cond 7	Cond 8
-	DMSO 10%	+	+	+	+	+	+	+	+
-	Glucose 5%	+	+	+	+	+	-	-	-
-	Z-VAD 60 µM	-	+	+	+	+	-	+	+
-	PVP 2%	-	-	+	+	+	-	-	+
-	ETG 1.5 M	-	-	-	+	+	-	-	+
-	0.2M Trehalose	-	-	-	-	-	+	+	+

Figure 5.12: Shows different activity of cryopreserved rat hepatocytes using UW with different CPAs (n=8). Results shows that; ***A) Hepatocytes viability*;** all conditions tested for hepatocytes cryopreservation maintained similar level of viability post thawing without any significant difference for the whole conditions, ***B) Overall activity MTT Assay:*** Overall activity of thawed hepatocyte showed that ***condition 6,7 and 8*** had significant higher overall activity (*** P=0.0009, **** P<0.0001) comparing with other conditions, ***C) Cell attachment SRB Assay:*** Hepatocyte attachment assay shows that ***condition 6,7 and 8*** had significant higher activity comparing to the other conditions **(***P=0.0142, **P=0.0031, **** P<0.0001).

D) Urea Assay: Hepatocyte cryopreserved with ***condition 6,7 and 8*** had significant higher level of urea production comparing to other conditions **(*****P=0.0002, **** P<0.0001). ***E) Cytochrome P450 EROD Assay (CYP 450 A1/2):*** Hepatocyte attachment assay shows that ***condition 6,7 and 8*** had significant higher Cytochrome P450 activity comparing to the other conditions ((*P=0.0224 COND1vsCOND7), (COND1vsCOND8 *P=0.0202), (COND1vsCOND6 ** P=0.0050)) ***and F) Albumin production ELISA Assay;*** shows that hepatocytes cryopreserved with ***condition 6,7 and 8*** had significant higher level of albumin production comparing to the other conditions **(****** P<0.0001)**.** the overall conclusion of hepatocyte cryopreservation with different cryoprotectants using UW solution suggested that suggested that combination of trehalose and Z-VAD enhanced the cryopreservation outcome.

5.4. Discussion and Conclusion

Hepatocyte transplantation is the promising alternative for the orthotopic liver transplantation (OTP) for the treatment of acute liver failure. Cryopreservation and banking of hepatocytes will be a good source of cells for the emergency.

In this part of my project, the first stages of developing hepatocyte cryopreservation protocol were carried out on hepatocytes, by testing/comparing seven different ready-made cryopreservation solutions. TC-Protector, Cell Banker1, CP1, Cryo-JIN, NutriFreez, pZever and University of Wisconsin (UW) solution.

Hepatocytes viability on thawing data showed significant differences between the 7 tested solutions. However, Cryo-JIN, Cell Banker and NutriFreez solutions gave similar cell viability post-thawing compared to UW solution. TC-Protector, CP1 and pZever solutions showed significant low viability comparing with other solution.

Cryopreserved hepatocytes overall activity showed that cells cryopreserved with UW, NutriFreez and Cell Banker1 gave similar activity level, however hepatocytes cryopreserved with TC-Protector, CP1 and pZever solutions showed significant low activity comparing with other solution. On the other hand, hepatocytes cryopreserved with Cryo-JIN showed significantly higher baseline overall activity compared to other cryopreservation solutions.

Cell attachment of fresh and cryopreserved hepatocyte tested at 24, 48 and 72 hours, showed that there was a slight decrease in hepatocytes attachment level of with time points. However, cells cryopreserved with Cryo-JIN showed significantly higher level of attachment compared to UW at baseline, were Cell Banker1, NutriFreez and CP1 gave smeller result of UW, on the other hand significant drop-in activity resulted when cryopreserved with TC-Protector and pZever solutions.

Urea production is another hepatocyte-specific function that is based on detoxification of ammonia. Cryopreservation results showed that hepatocytes cryopreserved in Cryo-JIN had the highest urea production level compared to other cryopreservation solutions. Were TC-Protector and pZever showed low level of urea production comparing to the other solutions, CP1, NutriFreez and Cell Banker1 had slightly higher level of urea production comparing with UW.

Cytochrome P450 (CYP450) activity in the liver is one of the essential functions for drug metabolism and toxins detoxification. Ethoxy resorufin-O-demethylase (EROD) assay is used to determine the level of induction of the xenobiotic-metabolizing enzyme cytochrome P-450 (CYP) 1A1/2 during the detoxification process by hepatocytes. Results showed that cryopreserved hepatocyte with UW, Ce1l Banker1, CP1 and NutriFreez solutions, were able to detoxify the xenobiotic agent through the activation of CYP 450 metabolic enzymes with the same level, however the activity of hepatocyte cryopreserved with TC-Protector and pZever were significantly lower than other solution and reduced by time, on the other hand Cryo-JIN maintained significantly higher activity level compared to all other solutions.

Albumin production is main function of hepatocytes, maintaining albumin production post cryopreservation is main goal of cryopreservation. Results showed that all fresh and cryopreserved hepatocytes were able to produce albumin but reduced by time, however all solutions showed significant lower level of albumin production except Cryo-JIN which maintained slightly higher level of albumin production comparing with whole other solutions.

The initial experiments of overall activity and cell attachment, ammonia detoxification (urea synthesis), cytochrome P450 and albumin production, showed that hepatocytes cryopreserved with Cryo-JIN produced a higher level of activity and production compared to other solutions indicating that Cryo-JIN could be a good hepatocyte cryopreservation solution.

The results obtained suggest that NutriFreez and Cell Banker1 solutions are giving similar results to UW solution, these cryopreservation solutions may be used instead of UW for hepatocyte microbeads cryopreservation and may give better results and will be tested in the next chapter for HMBs cryopreservation.

After testing the seven ready-made cryopreservation solution, more optimization was conducted on UW solution, since the initial data showed good cryopreservation outcome with (UW + 5% glucose + 10%DMSO). Eight different cryopreservation condition with different types and concentration of cryoprotectant were used to test their efficiency for hepatocyte cryopreservation, each contain different type of cryoprotectants (CPAs) and compared with Condition 1= UW + 5% glucose + 10%DMSO. Hepatocytes were cryopreserved with the eight different conditions and compared with condition 1, The results showed that addition of **Z-VAD** alone, and/ or **Trehalose** to UW solution improved the cryopreservation outcome.

Cryo-JIN showed better outcome in some assays of recovery; however, it was not possible to determine which ingredient(s) might have contributed to these positive effects as the manufacture was not willing to disclose the actual formulation of the product.

In conclusion the data obtained using seven ready-made solutions showed that other than UW, three solutions Cryo-JIN, NutriFreez and Cell Banker 1 could be used to cryopreserve hepatocytes to maintain good cell attachment, overall activity, urea production, and albumin synthesis, and drug metabolism functions. Moreover, WU solution supplemented with Z-VAD and Trehalose improved the cryopreservation outcome. Cryopreservation of rat hepatocyte microbeads using these solutions will be carried out in the next study, and the thawed assessed *in vitro* for cell viability and activity, and hepatocyte-specific function. Fresh rat hepatocyte microbeads will used as controls.

Chapter 6

Optimization of Hepatocyte Microbeads Cryopreservation

6.1. Introduction

6.1.1. Hepatocyte Microbeads Transplantation

Cryopreserved Hepatocyte Microbeads have been tested for its efficiency to cure acute liver failure ALF and different liver diseases. Intraperitoneal transplantation of hepatocyte microbeads has been successfully established at King's College Hospital London for treatment of children with acute liver failure. The microbeads used were freshly produced at the time of transplantation. It would be ideal if the microbeads are available, especially for emergency cases. This could only be achieved by establishing banked cryopreserved microbeads. Therefore, optimizing a protocol for hepatocyte microbeads cryopreservation is essential. Initial study of hepatocyte microbeads cryopreservation at King's (Jitraruch et al., 2014) using UW solution and controlled-rate freezer. The outcome of this study showed some encouraging results, however, further modifications of the protocol used are required.

In a study done by Baldini et al. (2008) to assess the viability and function of cryopreserved porcine hepatocyte microbeads, hepatocyte microbeads were cryopreserved at 196ºC for one month, then transplanted at rat peritoneal cavity without immunosuppression. The hepatocyte microbeads were left for one month in the rat peritoneal cavity, one month later microbeads were explanted and assessed for function and viability. The results showed that long term cryopreservation of hepatocyte microbeads resulted in retention of their biological activity (Baldini et al., 2008).

Aoki et al. (2005) reported a study on cryopreservation of encapsulated hepatocytes (human and rat) in alginate/poly-L-lysine. The hepatocyte microbeads were cryopreserved using DMEM medium supplemented with 10% FBS and 10% DMSO and frozen immediately

in liquid nitrogen. One month later, cryopreserved human hepatocyte microbeads were transplanted in rat spleen, without immunosuppression. The transplanted cryopreserved HMBs showed good maintenance of both cell survival and viability in rat spleen. They suggested that due to shortage of donor organs, cryopreservation of encapsulated hepatocytes may be a solution for this issue, and will contribute to the success of hepatocyte transplantation.

In 2009, Mei et al. cryopreserved encapsulated pig hepatocytes and transplanted in a mouse model of fulminant liver failure. The study showed that cryopreserved encapsulated hepatocytes were functional and survived for two weeks post-transplantation (Mei et al., 2009). Moreover, a study by Sgroi et al. (2011) showed that intraperitoneal transplantation of encapsulated human hepatocytes in mice with ALF, resulted in a significant survival of the mice and liver regeneration that restored the missing liver functions (Sgroi et al., 2011).

As mentioned earlier hepatocyte transplantation team at King's College Hospital are the first team among the world who initiated and developed hepatocyte microbeads transplantation in human. It is being used for treatment of children with ALF, the technique involves the interpertonial transplantation of human hepatocyte microbeads under ultrasound guidance (Dhawan et al., 2020). Due to the luck of donors and the shortage availability of the fresh hepatocytes, and low viability of the cryopreserved hepatocytes post thawing, and the long process of microbeads production, hepatocyte microbeads bio-banking could solve these problems especially for the new borne with hepatic failure.

6.1.2. Alginate encapsulated hepatocyte transplantation in ALF

As mentioned above King's was the first center in the world used intraperitoneal human hepatocyte microbeads (HMBs) transplantation in children with ALF, without the need for immunosuppression. The team reported the safety and feasibility of this work, and the outcome was very encouraging. More patients are being recruited for such treatment; therefore, production of human hepatocyte microbeads will be in demand. Cryopreserved and banked HMBS cryopreservation could help in sorting out shortage of donor liver and will be available for immediate transplantation especially in emergency cases.

In this chapter the hepatocyte microbeads cryopreservation protocol is being optimized by testing commercially available cryopreservation solutions with/without the addition of cryoprotectants. The thawed hepatocyte-specific functions and activity will be assessed *in vitro* and compared to fresh microbeads as controls.

6.2. Materials and Methods

6.2.1. Chemicals and Solutions for Hepatocyte Microbeads Production and Assays

All chemicals and solution used for microbeads preparation and assays are listed in Chapter 2 Materials & Methods.

6.2.2. Experimental Design

Produced HMBs were cryopreserved using the seven different readymade, commercially available cryopreservation solutions based on their effectiveness for hepatocyte cryopreservation used earlier in **Chapter (5)**. All solutions were tested and compared with UW solutions supplemented with 10% DMSO and 5% glucose. Ratio of 1:2 microbeads to cryopreservation solution were used, i.e., for each 1ml of microbeads, 2ml of cryopreservation solution were added using 50 ml cryo-bags. Therefore, the addition of microbeads to cryopreservation solution results in dilution of DMSO from 10% to about 6.66%, and glucose from 5% to 3.3%.

After testing the readymade cryopreservation solution for HMBs cryopreservation, HMBs were cryopreserved with Cryo-JIN and UW solutions supplemented with different cryoprotectant using Vitrification methods, finally the solutions which gave best cryopreservation results on hepatocytes were tested on HMBs and analyzed for hepatocyte specific activity.

6.2.3. Hepatocyte Encapsulation

Freshly isolated hepatocytes were checked for cell number and viability, then mixed with 1.5% NovaMatrix SLG20 alginate prepared as described in Chapter 2, Section (2.2.12.1). Encapsulation process was conducted as described in Chapter 2, Section (2.2.12.2).

6.2.4. HMBs Cryopreservation Using Slow Freezing Method Controlled-Rate (CRF)

Hepatocyte microbeads were washed with EMEM, all steps were proceeded on ice, cryopreservation solutions with different cryoprotectants selected for hepatocyte microbeads work at a ratio of 1:2 (microbeads and cryopreservation solution). Controlled-Rate Freezer (CRF; model: Kryo10 from Planer, UK) was programed with the freezing protocol shown in **Table (6.1)**, then the frozen samples were transferred to -140 ºC freezer and stored for two weeks.

Table 6.1: Controlled-Rate Freezer Program for HMNBs Cryopreservation

Step	Start Temperature	Rate	Time	End Temperature
1	0ºC	Hold	8 min	0ºC
2	0ºC	-2ºC/min	4 min	-8ºC
3	-8ºC	-35ºC/min	6 Sec	-28ºC
4	-28ºC	-2.5 ºC/min	2 min	-33ºC
5	-33ºC	+2.5 ºC/min	2 min	-28ºC
6	-28ºC	-1 ºC/min	16min	-60 ºC
7	-60 ºC	-10 ºC/min	4 min	-100 ºC
8	-100ºC	-20 ºC/min	3min	-160 ºC

6.2.5. HMBs Cryopreservation by Vitrification

Cryopreservation with vitrification was tested using Cryo-JIN cryopreservation solutions, and UW with different cryoprotectants listed in in **Table (6.2)**. 10 ml of produced microbeads were resuspended in cryopreservation solution and transferred into cryo-bags using a 50ml syringe fitted with a Kwill®. Bags were sealed using a tube sealer, then rapidly snap frozen by immediate placing in liquid Nitrogen for 1 hour. Bags were transferred to -140 ºC freezer.

Table 6.2: vitrification solutions

Vit Cond1	Vit Cond2	Vit Cond3	Vit Cond4
- UW - 10% DMSO - 5% Glucose	- UW - 5 % Glucose - 15% DMSO - 15%Glycerol	- UW - 10% DMSD - 16.83% M ETG - 0.6 M Trehalose - 60 µM Z-VAD	- Cryo- JIN

[**Vit**=Vitrification]

6.2.6. Final HMBs Cryopreservation Condition

After testing different cryopreservation solution with different cryoprotectants, the best results were obtained when using Cryo-JIN and UW solution with the addition of trehalose and Z-VAD therefor final testing of HMBs optimisation was carried on the following condition listed in **Table (6.3)**.

Table 6.3: Final HMBs Cryopreservation Conditions using CRF

Cond 1	Cond 2	Cond 3
- UW - 10% DMSD - 5% glucose	- UW - 10% DMSD - 1.5% M ETG - 0.2M Trehalose - 60 µM Z-VAD	- Cryo-JIN

6.2.7. Hepatocyte Microbeads Culturing

Fresh and thawed microbeads were cultured by using Phenol red free William's E medium (WEM), supplemented with heat-inactivated fetal calf serum 10% FCS, 10 mM HEPES, 2mM L-glutamine, 0.1µM Dexamethasone, 5 ml ITS, penicillin 50 U/ml and streptomycin 50 µg/ml., microbeads were incubated at 37 ºC and 5% CO_2 incubator and tested for its activity on Day 1, 3, and 7 respectively.

6.2.8. Hepatocyte Microbeads Activity Assays

All assays were conducted as described in Chapter 2, Hepatocyte Overall Metabolic Activity (**MTT Assay**; Section 2.2.14), Albumin (ELISA Assay; Section 2.2.15), Ammonia detoxification (**Urea Assay**; Section 2.2.16), and Cytochrome P450 (**EROD Assay**; CYP 450 A1/2; Section 2.2.17).

6.2.9. HMBs Viability Determination Using FDA/PI

Hepatocyte viability within the microbeads was detected using FDA/PI staining. 10µl of 1mg/ml FDA and 20µl of 1mg/ml PI were added to 1 ml of microbeads suspension for 90 sec in the dark, and then washed two times with DPBS. Microbeads were then re-suspended in 250µl DPBS. A sample of stained microbeads were placed onto a microscope glass slide. Cells were visualized under fluorescent microscope. Live cells cytoplasm stained green **(FDA)**, while the nuclei of dead cells stained red **(PI)**.

6.3. Results

6.3.1. HMBs Slow Freezing Method using CRF

The seven readymade cryopreservation solution listed in **Table (5.1)**; **C**hapter 5 have been tested for use in hepatocyte microbeads cryopreservation. Initially, empty microbeads were cryopreserved using CRF to assess the effect of these solutions on empty SLG-20 microbeads, to determine the effect of solution on alginate, then HMBs were cryopreserved suing the same solutions. Each solution resulted in a different cell viability, and microbead morphology as shown in **Figure (6.1)**.

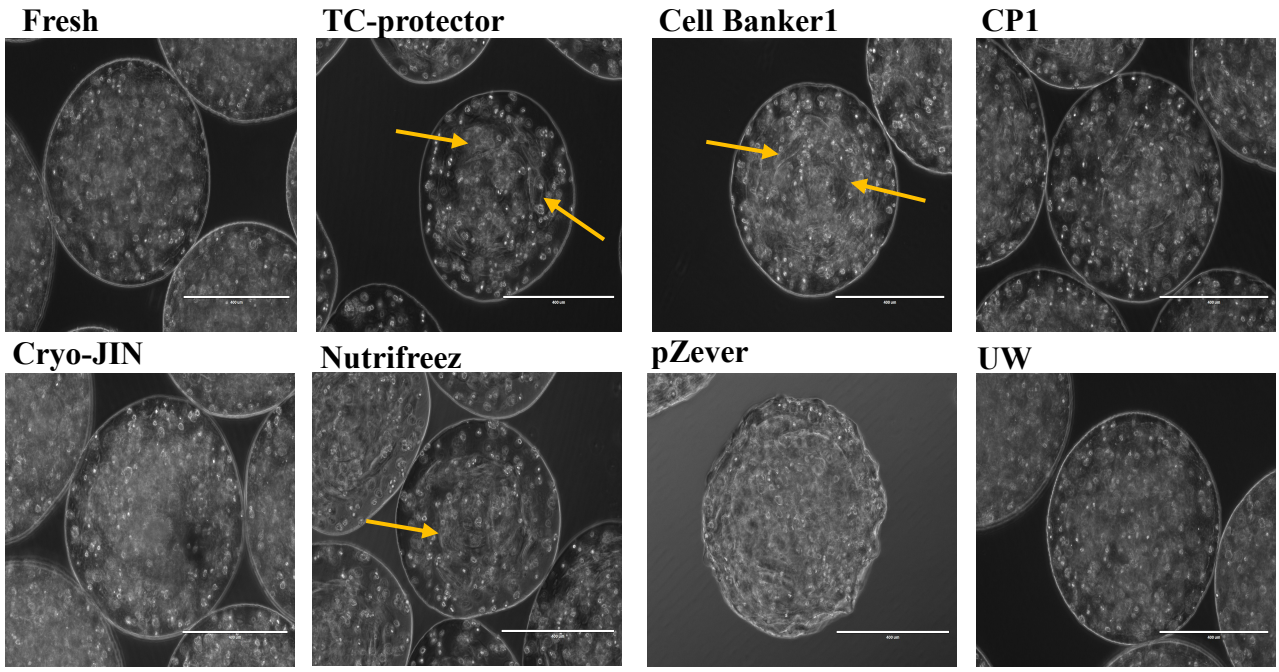


Figure 6.1: Images of thawed hepatocyte microbeads, showing the effects of ready-made cryopreservation solution on microbeads morphology. Damage to the alginate structure could be observed in TC-protector, Cell Banker, and NutriFreez thawed HMBs (yellow arrows). This could have happened as a result of ice formation within the microbeads during cryopreservation process. however cryopreserved microbeads using pZever resulted in changes in the microbeads shape and size. Microbeads cryopreserved using Cryo-JIN, CP1 and UW maintained their shape and morphology.

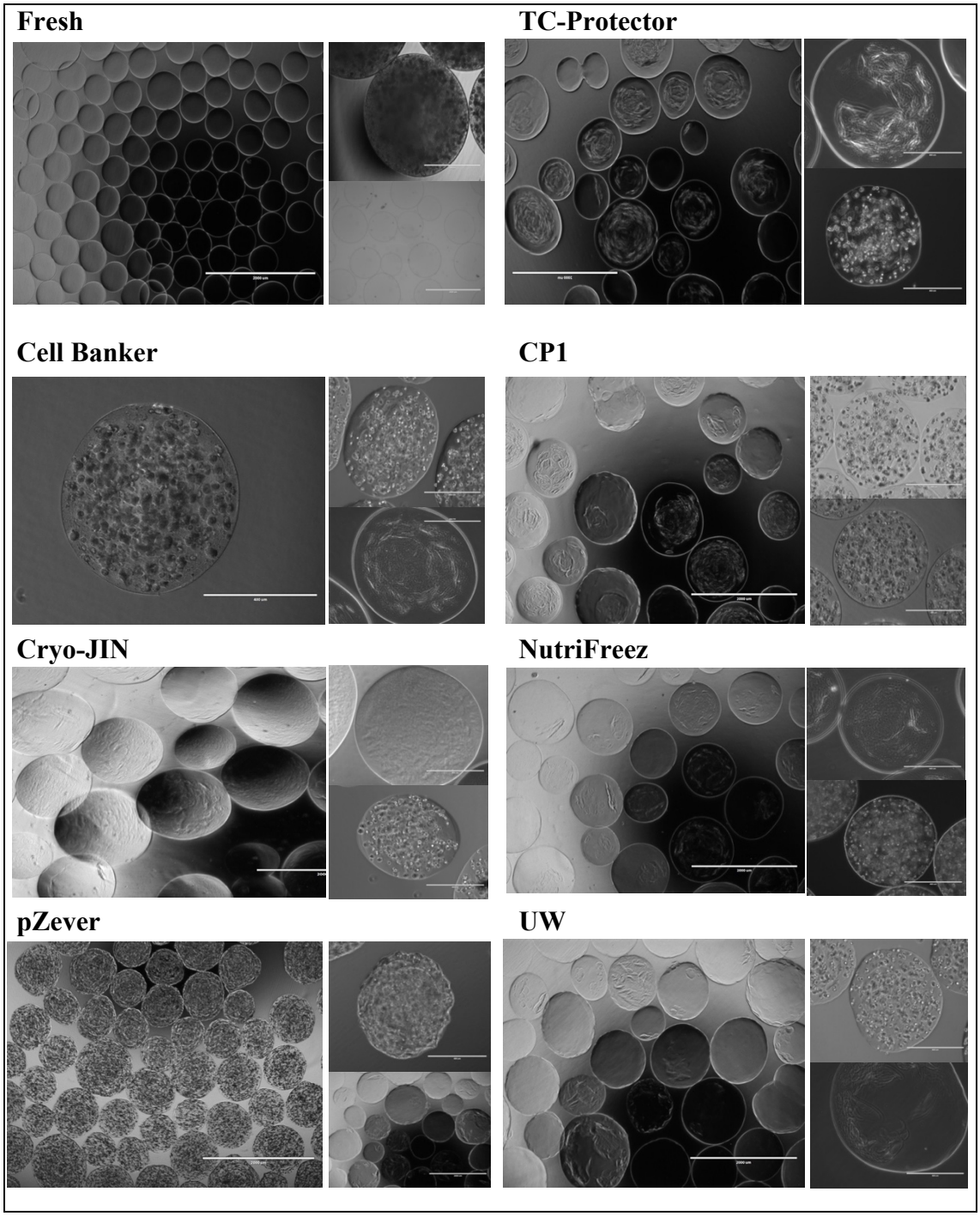


Figure 6.2: Empty Microbeads, and HMBs morphology post-thawing. Microbeads were cryopreserved using CRF and stored for 7 days. Empty Alginate microbeads have been produced and cryopreserved using the ready-made cryopreservation solutions, before testing them in hepatocyte cryopreservation. To determine if the solutions with the cryopreservation process could cause any physical effects on the microbeads. Then the same solutions have been tested for HMBs cryopreservation.

6.3.2. Human Hepatocytes Microbeads Cryopreservation Using Readymade Solution

Human Hepatocytes were isolated from liver tissues unused for transplantation (n=11), hepatocytes were encapsulated with 1.5% SLG-20 alginate using Inotech-ER encapsulator IE-50R. HMBs then washed and prepared for cryopreservation, using the same seven earlier mentioned ready-made cryopreservation solutions. HMBs were mixed with the cryopreservation and placed at 50 ml Cryo-bags. HMBs were cryopreserved using CRF and vitrification, then samples were shifted to -140°C freezer. After one-week HMBs were thawed, then cultured and tested for its viability and activity.

First fresh HMBs were cultured at 37°C incubator and tested for its activity and viability, then cryopreserved HMBs were thawed, washed with DPBS at room temperature with 1:2 volume of DPBS to HMBs. HMBs were then cultured and tested on day 1, 3, and 7 for its activity and viability. Data (n=11) are presented as mean ± SD. Two-way ANOVA test was used to compare the different solutions, and multiple comparisons using UW as a control. A P-value of <0.0001 was considered as statistically significant.

6.3.2.1. HMBs Viability Using FDA Stanning

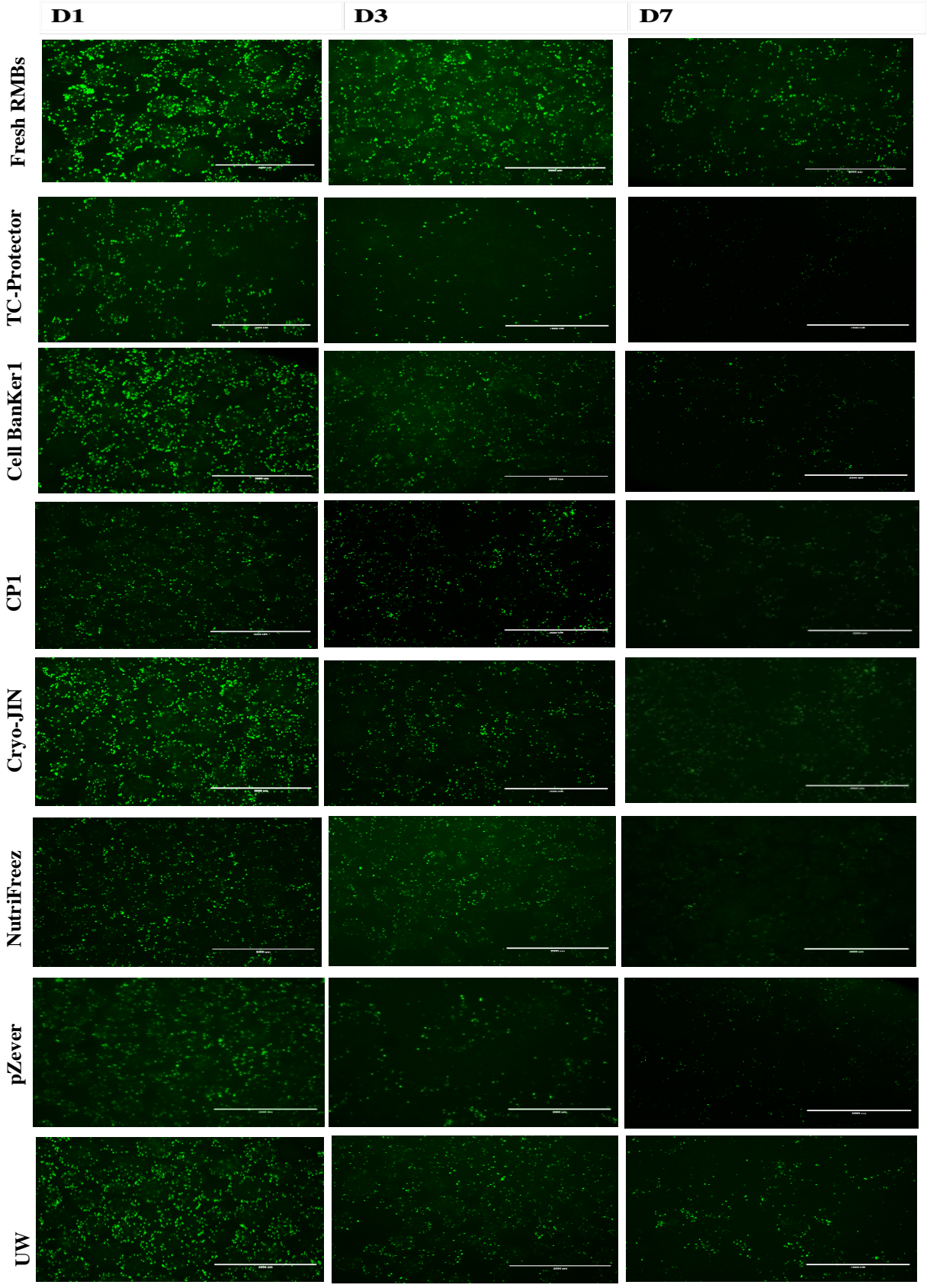


Figure 6.3: Hepatocyte viability in cultured RMBs post-thawing using FDA staining only.

6.3.2.2. Human Hepatocyte Microbeads Overall Metabolic Activity- MTT Assay

The overall metabolic activity of hepatocytes within the microbeads HMBs were assessed by MTT assay, healthy and viable hepatocyte should be able to reduce MTT (yellow) to formazan (purple), which is measured by spectrophotometer, which reflect the activity of the living hepatocyte within the microbeads as presented in **Figure (6.4)**.

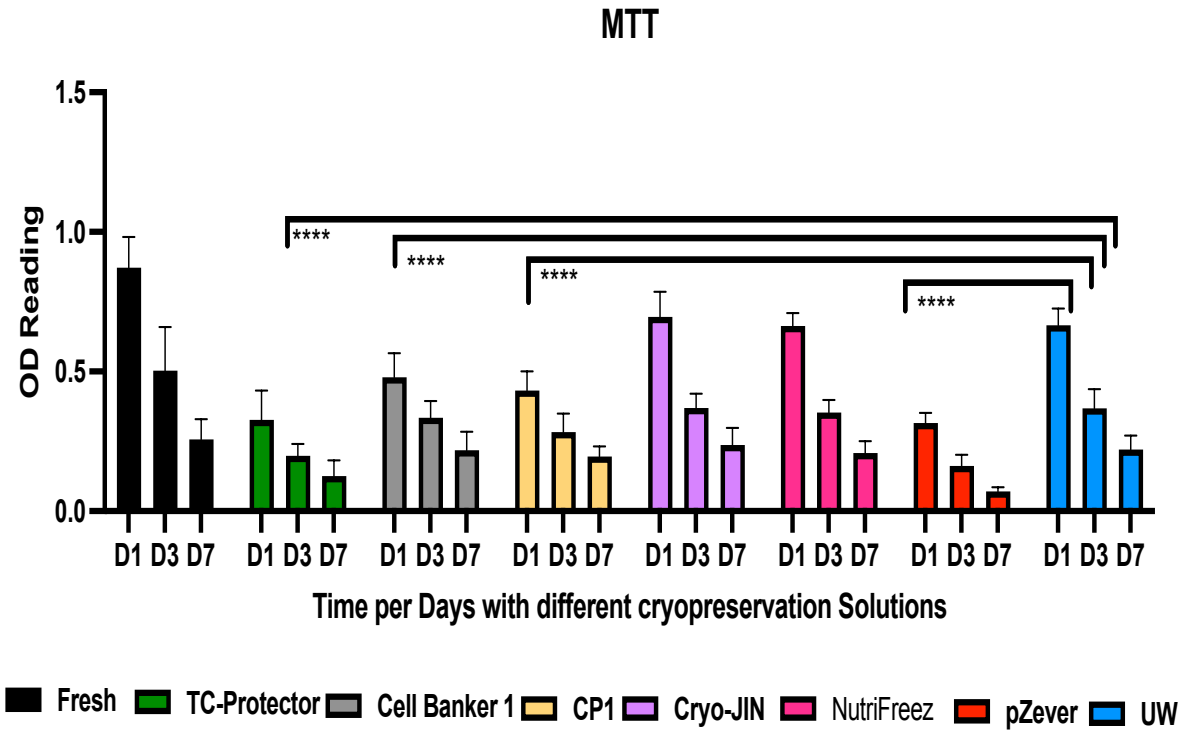


Figure 6.4: MTT results of fresh and cryopreserved human hepatocyte microbeads (n=11). The results shows that overall metabolic activity of HMBs decreased with time. HMBs cryopreserved in UW, NutriFreez and Cryo-JIN showed similar activity level, however hepatocytes cryopreserved with Cell Banker1, TC-Protector, CP1 and pZever solutions showed a statistically significant lower activity compared to other solution. [****P: <0.0001; UW = University of Wisconsin].

6.3.2.3. Human Hepatocyte Microbeads Urea Assay – Ammonia detoxification

Urea assay is colorimetric assay used to determine the urease enzyme activity, by challenging the hepatocytes with Ammonia, living hepatocyte will be able to detoxify the ammonia and urea will be produced as by product which can be easily measured by measuring the level of urea production. Living hepatocytes within microbeads should be able to detoxify the Ammonia, HMBs were tested for urea production, and data are presented in **Figure (6.5)**.

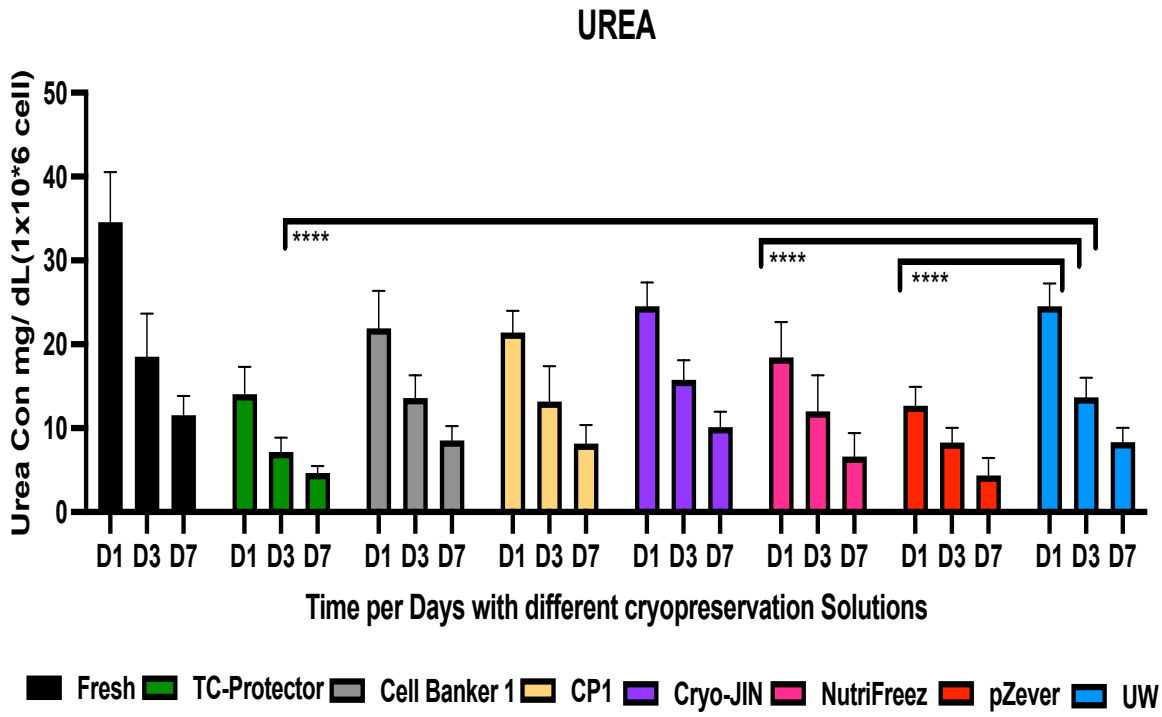


Figure 6.5: Urea assay of cryopreserved human hepatocytes microbeads; showed that hepatocytes were able to detoxify ammonia and produce urea, however the level of the production decreased with time. HMBs cryopreserved using Cell Banker1, CP1and Cryo-JIN had similar level of urea production to UW solution. On the other hand, NutriFreez, pZever and TC-Protector solutions showed a statistically significant decrease in levels of urea production compared to other solutions [****P:<0.0001; UW = University of Wisconsin].

6.3.2.4. Human Hepatocyte Microbeads Cytochrome P450 EROD Assay (CYP 450 A1/2)

One of the main functions of hepatocyte is to detoxify the body from any toxic or xenobiotic agent that enter the body, through the activation of Cytochrome P450 monooxygenase 1A (CYP 1A) enzyme. HBMs were induced by Omeprazole, then tested for EROD activity on day 1, 3, and 7; data are presented in **Figure (6.6)**.

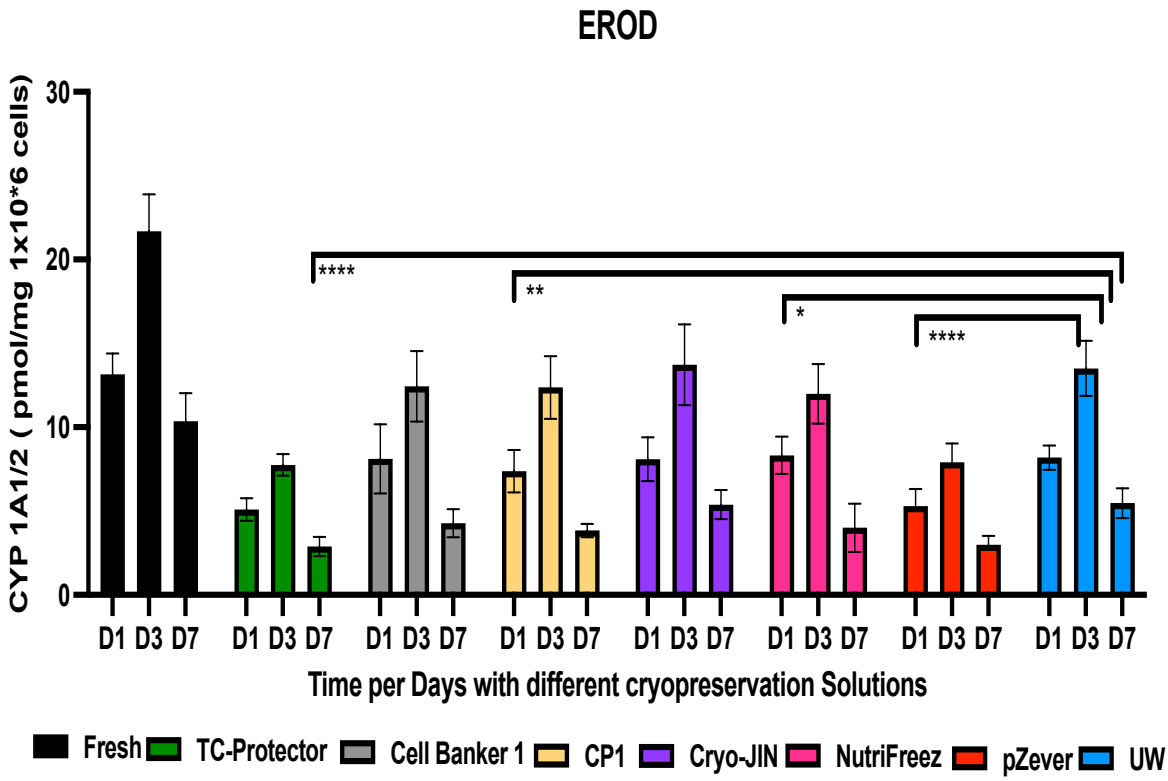


Figure 6.6: EROD assay of cryopreserved human hepatocyte microbeads; results shows that HMBs cryopreserved with UW, Ce11 Banker1 and Cryo-JIN were able to detoxify the xenobiotic agent through the activation of CYP 450 enzymes with similar levels, however the activity of hepatocytes cryopreserved with TC-Protector, CP1, NutriFreez and pZever were of statistically significant lower level than control solution, UW [****P:<0.0001; **P: 0019; *P: 0.0206; UW = University of Wisconsin].

6.3.2.5. Human Hepatocyte Microbeads Albumin Production - ELISA Assay

Albumin synthesis is one of the main function of hepatocytes, Hepatocytes within the microbeads were tested for its ability to produce albumin; data are presented in **Figure (6.7)**.

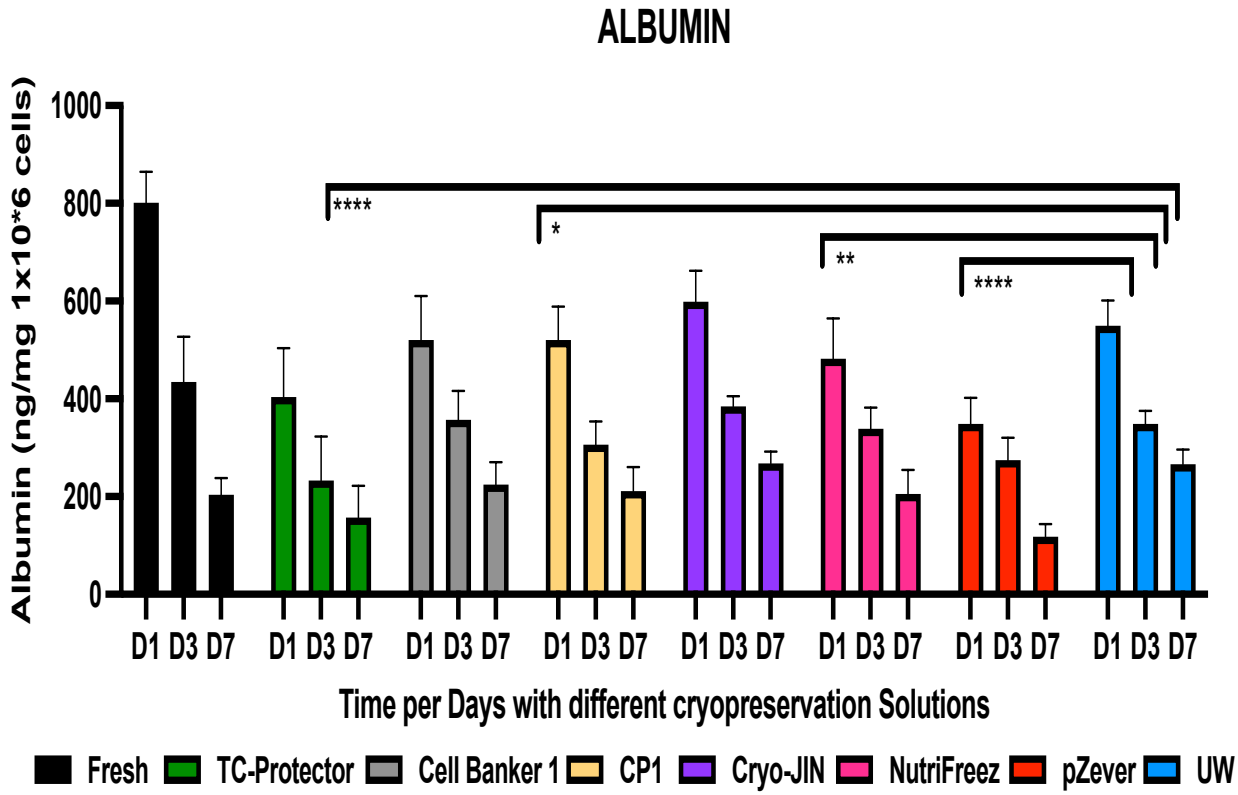


Figure 6.7: ELISA Assay for Albumin production by cryopreserved human hepatocyte microbeads, shows that microbeads cryopreserved using Cell Banker1, Cryo-JIN and UW solution produced similar levels of albumin, however HMBs cryopreserved using CT-Protector, CP1, NutriFreez and pZever produced of albumin at lower levels compared to UW solution. [****P:<0.0001; **P: 0083; *P: 0.0183; UW = University of Wisconsin].

6.3.3.1. Rat Hepatocyte Microbeads Cryopreservation using Readymade Solution

The same earlier experiments were repeated on rat hepatocytes (n=8) for more verification. Results are presented bellow with overall activity Assay (MTT), Urea production, EROD activity and Albumin synthesis.

6.3.3.2. Rat Hepatocyte Microbeads Overall Metabolic Activity- MTT Assay

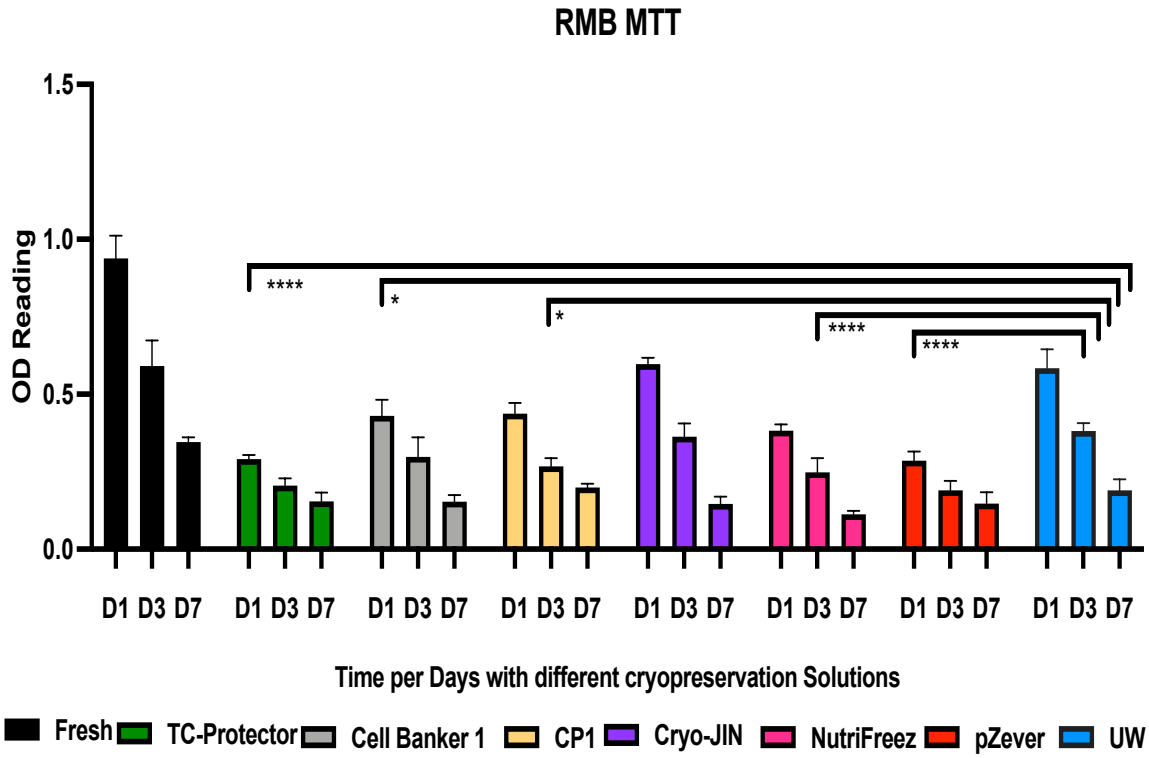


Figure 6.8: The overall activity data for cryopreserved RMBs; results shows that only Cryo-JIN maintained similar activity to UW solution, while all other solutions showed a decrease in the activity with time compared to UW solution [****P:<0.0001; UW vs. Cell Banker 1: *P: 0100; UW vs. CP1: *P: 0.0203; UW = University of Wisconsin].

6.3.3.3. Rat Hepatocyte Microbeads Urea Assay – Ammonia detoxification

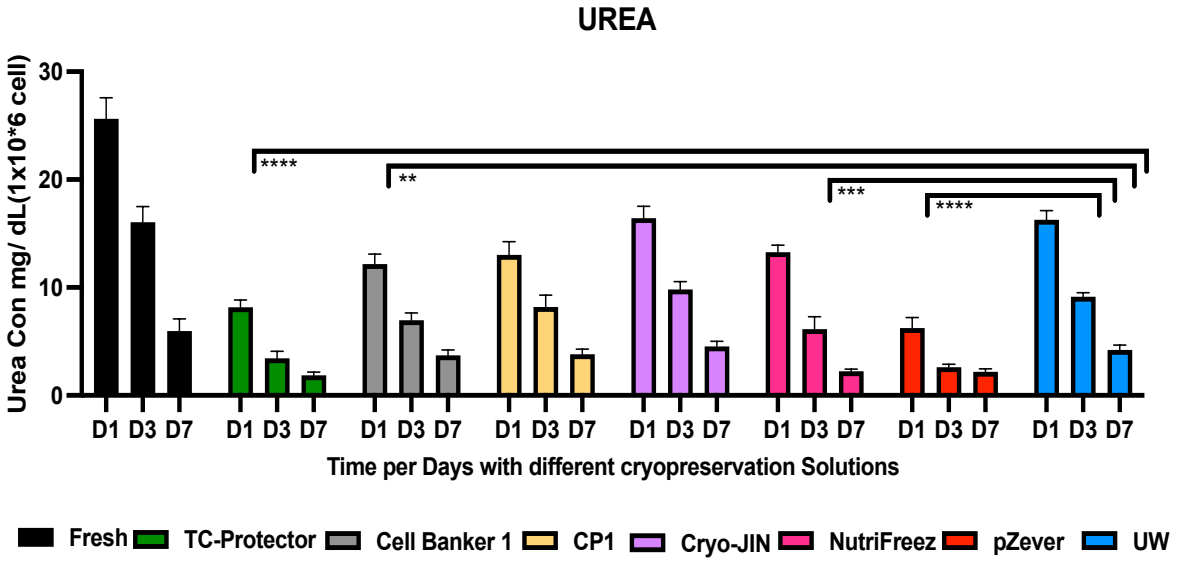


Figure 6.9: Urea assay of cryopreserved RMBs; results shows that CP1 and Cryo-JIN solution gave similar values compared to UW solution, while TC-Protector, Cell Banker1, NutriFreez and pZever gave statistically significant lower values compared the control solution UW. [****P:<0.0001; ***P: 0.002; **P: 0.0022; UW = University of Wisconsin].

6.3.3.4. Rat Hepatocyte Microbeads Cytochrome P450 EROD Assay (CYP 450 A1/2)

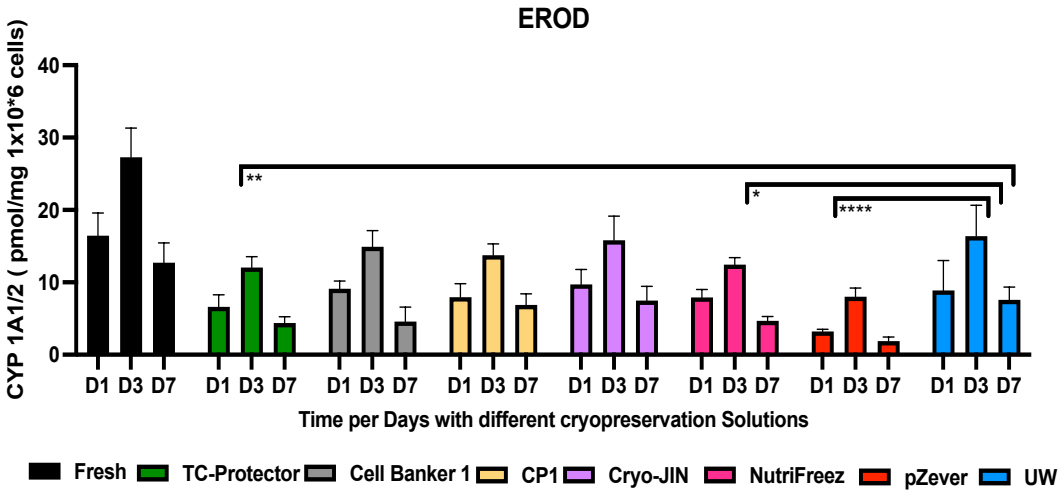


Figure 6.10: EROD assay of cryopreserved RMBs; results shows that Cell Banker1, CP1 and Cryo-JIN gave similar results compared to control solution, UW, while TC-Protector, pZever and NutriFreez shows statistically significant lower activity. [****P:<0.0001; **P: 0011; *P: 0.0129; UW = University of Wisconsin]

6.3.3.5. Rat Hepatocyte Microbeads Albumin Production - ELISA Assay

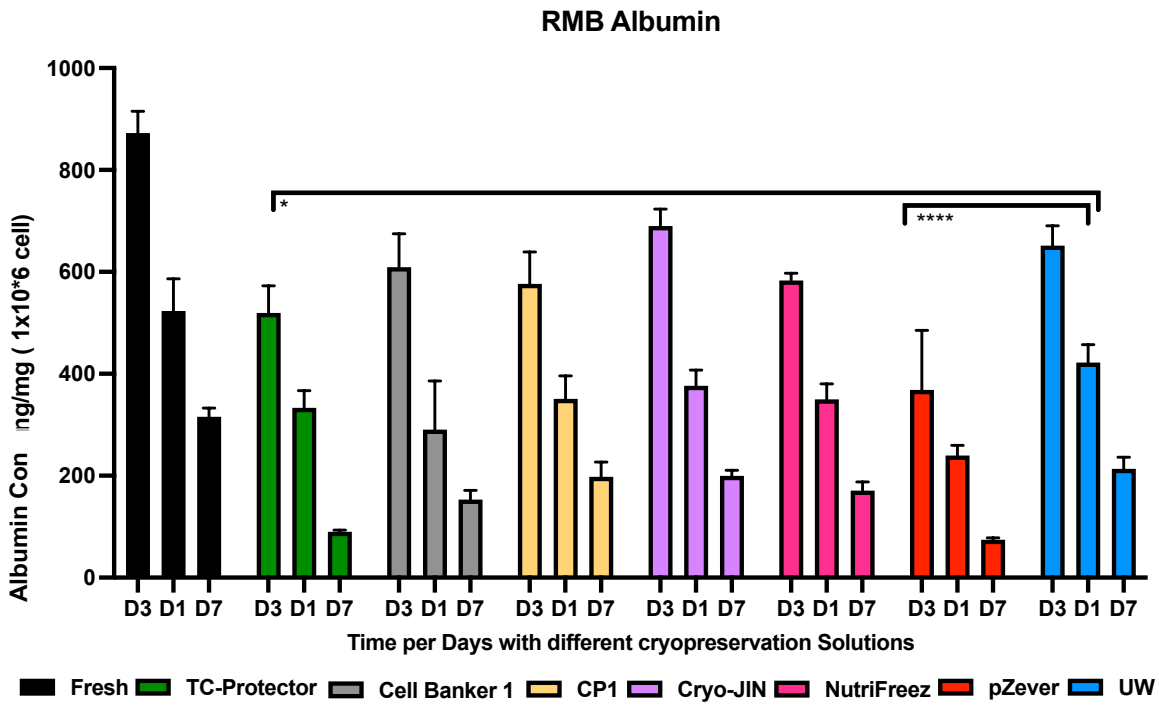


Figure 6.11: Albumin synthesis by cryopreserved RMBs shows that most of the ready-made cryopreservation solutions were able to produce albumin at levels similar to UW solution except TC-Protector and pZever showed statistically significant drop in albumin synthesis compared to UW solution. [****P:<0.0001; *P: 0.0183; UW = University of Wisconsin].

6.3.4. Vitrification of Human Hepatocyte Microbeads

Quick freezing method or vitrification have been used to cryopreserve variety types of cells and tissues, and many cryopreservation protocols have been developed to avoid cryo-damage and ice formation. in this part of my project cryopreservation with vitrification was tested with Cryo-JIN cryopreservation solution since it shows good slow freezing results, also UW have been tested with different cryoprotectants as described in **Table (6.2)**.

Vitrification of HMBs results showed that all cryopreservation solutions resulted in broken or cracked microbeads as shown in **Figure (6.12)**, this could happen due to the high content of water in the alginate or due to other reasons which should be studied further. Moreover, vitrification success depends on very fast cooling and warming, and as 50 ml bags were used in these experiments, therefore, transfer of heat might have been slow leading to damage to microbeads. Further testing of this technique using small volumes of microbeads/cryopreservation solution may results in a better outcome.

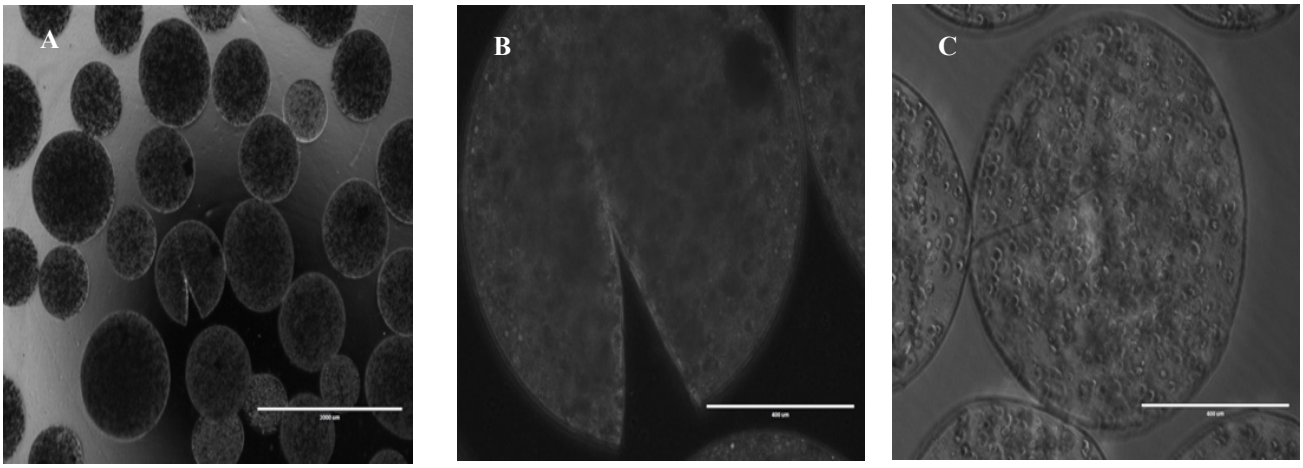


Figure 6.12: Images of HMBs cryopreserved using vitrification under light microscope. **A)** Random field showing microbeads after thawing. **B)** Representative image of a broken microbead. **C)** A representative image of an intact microbead with a hairline crack in it.

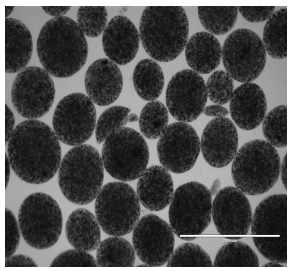
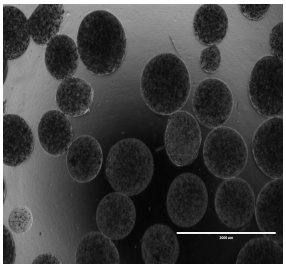
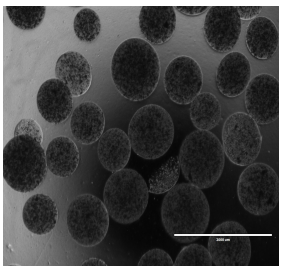
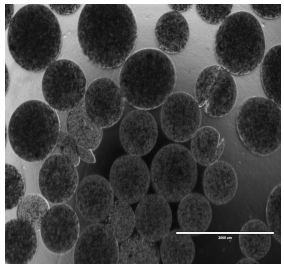
V cond1	V cond2	V Cond3	V Cond4
			
- UW - 10 % DMSO - 5% Glucose	- UW - 5 % Glucose - 15 % DMSO**** - 15 %Glycerol*	- UW - 10% DMSD - 16.83% M ETG** - 0.6 M Trehalose*** - 60 μM Z-VAD	- Cryo- JIN

Figure 6.13: Representative images of vitrification outcome of various conditions, results show damage to HMBs (cracked or broken) [**V** = vitrification].

*Royere et al., (1996).
** Fahy et al.,(2004).
*** Martinetti et al., (2017).
**** J. H. Lee, Jung, Lee, Park, & Lee, (2012).

6.3.5. Rat Hepatocytes Microbeads Final Cryopreservation Conditions

After testing different cryopreservation solution with different cryoprotectants for hepatocytes and HMBs, the best cryopreservation outcomes were obtained when using Cryo-JIN and UW solution with the addition of trehalose and Z-VAD therefor final testing of RMBs cryopreservation optimisation was tested on the following condition listed in **Table (6.3)**, experiments were repeated with (n=8). RMBs were cryopreserved using CRF. One week post cryopreservation RMBs were thawed, cultured, and tested for viability and Activity.

Table 6.3: Final RMBs cryopreservation Conditions using CRF

Cond 1	Cond 2	Cond 3
- UW - 10% DMSD - 5% glucose	- UW - 10% DMSD - 1.5 M ETG* - 0.2M Trehalose - 60 µM Z-VAD	- Cryo-JIN

6.3.6. RMBs Viability Determination FDA Stanning

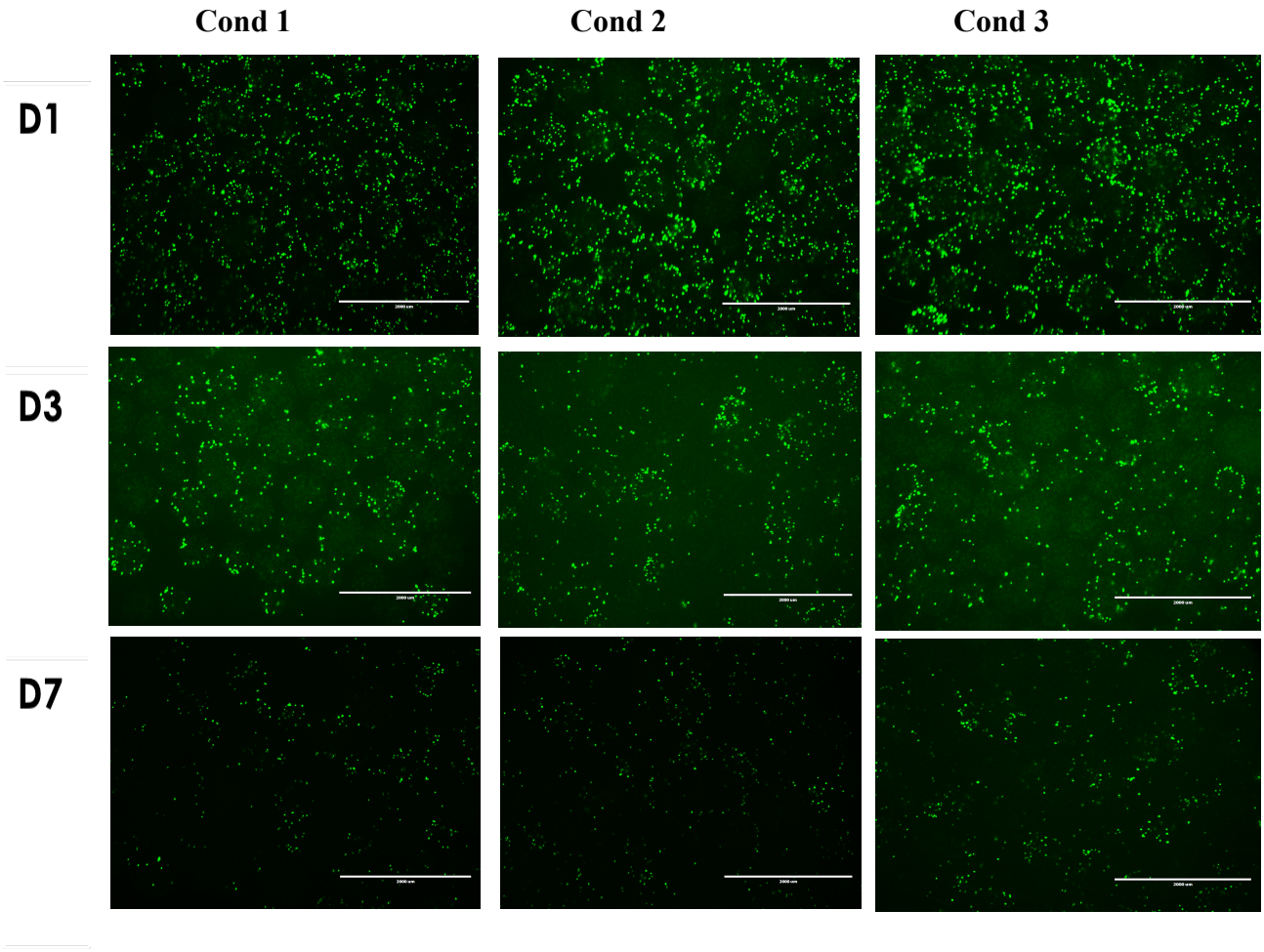


Figure 6.14: FDA staining of cultured cryopreserved RMBs **Cond1**, **Cond2**, and **Cond3** solutions on Day 1, 3 and 7, RMBs thawed one week post cryopreservation. All conditions showed good viability, however cell viability dropped with time.

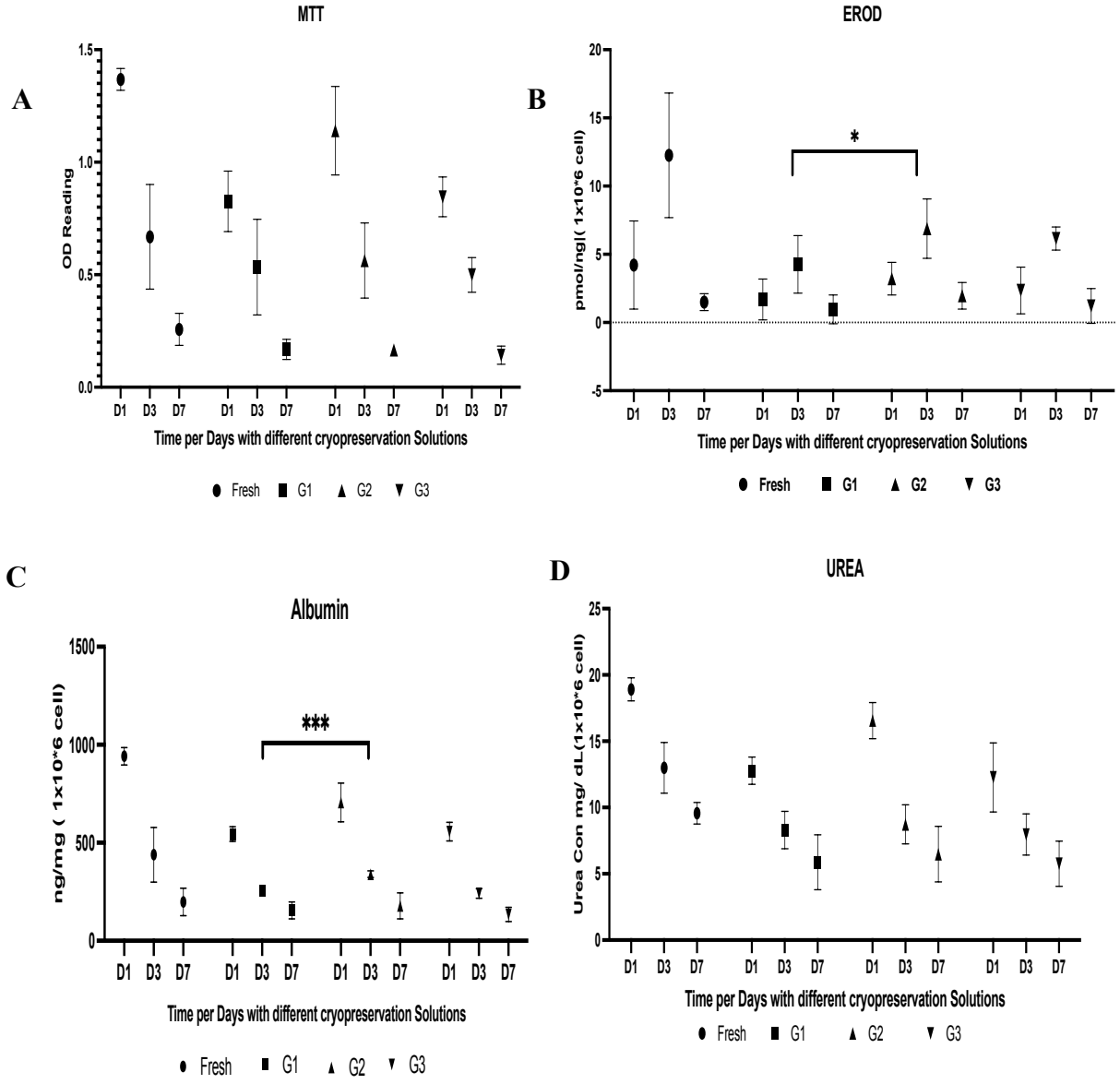


Figure 6.15: A) Shows the overall activity of **Cond 1, 2** & **3** were all solution gave same level of activity without any significant difference; **B)** Shows EROD assay results, there was a slight higher and statistically significant level of activity with Cond 2 vs Cond 1 [* P: =0.0179]; **C)** Albumin synthesis data, where Cond 2 shows a statistically significant higher level of albumin synthesis compared to other groups [***P: =0.0003]; **D)** Urea production, all conditions gave similar urea production levels.

6.4. Discussion and Conclusion

Optimization of cryopreservation protocol for HMBs is the main goal of this project, enabling such protocol will assist the biobanking of HMBs for future clinical needs.

In this chapter, HMBs were produced and cryopreserved with the same seven different ready-made cryopreservation solutions tested on hepatocyte cryopreservation in Chapter 5TC-Protector, Cell Banker1, CP1, Cryo-JIN, NutriFreez, pZever and University of Wisconsin (UW) solution.

First empty microbeads were cryopreserved with the seven different solutions to test the morphology and the effect of the solution on HMBs. Then HMBs were cryopreserved with the same solutions. The results showed that viability of HMBs by using FDA/PI stanning, was variable between the solution, however there were general decrease in the viability of the HMBs by day 7, where most of the hepatocytes were dead as illustrated in **Figure (6.3)**.

Cryopreserved HMBs with the seven-solution showed that HMBs overall activity, Cytochrome P450 (CYP450) activity, Albumin synthesis and urea production when using UW, Cryo-JIN and Cell Banker1 gave similar activity level, however HMBs cryopreserved with TC-Protector, CP1, NutriFreez and pZever solutions showed significant low activity comparing with other solution, in consequence these four solutions are not recommended for HMBs cryopreservation. On the other hand, in most experiment Cryo-JIN maintained slightly higher level of activity comparing with whole other solutions, Therefore Cryo-JIN is recommended for HMBs cryopreservation. However, further studies are needed to validate Cryo-JIN efficacy for clinical use. This depends on the suitability of its formulation and ingredients for cryopreservation of clinical grade microbeads.

Based on the earlier results of hepatocyte cryopreservation using UW solution with addition of Z-VAD and Trehalose, HMBs cryopreservation was tested by using; UW solution with (10%DMSO, 1.5% M ETG, 0.2M Trehalose and 60 μM Z-VAD) and compared with (UW

+ 5% glucose + 10%DMSO) and Cryo-JIN. The results showed that WU and Cryo-JIN solution gave similar cryopreservation outcomes, however UW solution supplemented with Z-VAD, ETG and Trehalose resulted with slightly higher activity.

In addition to slow cryopreservation method using the CRF, HMBs cryopreservation were tested using the vitrification method. FDA/PI stanning showed that HMBs was viable post vitrification, but unfortunately some cracks were witnessed in some microbeads, which is unfavorable for the transplantation purpose, therefor HMBs vitrification using the condition listed in **Table (6.2)** is not accepted. However, vitrification of encapsulated HepG2 spheroids was possible using a different vitrification approach that could be used in the future to cryopreserve alginate-encapsulated cells (Fuller et al., 2017).

After testing the seven ready-made cryopreservation solution, and the optimized UW solution with different addition and concentration of cryoprotectant, I conclude that Cryo-JIN and Cell Banker is recommended for hepatocyte and HMBs cryopreservation. In addition, UW solution with the addition of **Z-VAD** alone and or **Trehalose** to UW solution improved the cryopreservation outcome.

In conclusion the data of hepatocyte cryopreservation using readymade solutions showed that UW, Cryo-JIN and Cell Banker 1 resulted in good hepatocyte overall activity, urea production, albumin synthesis, and drug metabolism functions post-thawing. These findings suggest that HMBs could be cryopreserved using these solutions.

However, the addition of **Z-VAD** and **Trehalose** to UW solution improved the cryopreservation outcome. Therefore, UW supplemented with these CPAs could be the “ideal” mixture for cryopreservation of hepatocytes and HMBs. Further studies are needed to assess the efficacy of transplantation of hepatocyte microbeads cryopreserved using the above tested solutions in an acute liver failure animal model, compared to fresh microbeads.

Chapter 7

Apoptosis in Cryopreserved Hepatocytes

7.1. Introduction

Apoptosis and necrosis post-cryopreservation are the main challenges facing cryobiologist. Developing cryopreservation protocols and studying the effects of cryoprotectants on cells during cryopreservation process and thawing helped scientists to understand the mechanism of ice formation and preventing cells death during cryopreservation (Martin et al., 2004). There are many factors that should be considered to get best outcome of cryopreservation such as cell type, pre-Freezing processing, cryoprotectants, cryopreservation solutions, cryopreservation (freezing) protocol, cell storage (banking), and thawing protocols. All these factors could affect cell viability during and post-cryopreservation, and on thawing too. Therefore, studying all these factors will help in getting best cryopreservation outcome and avoiding cell damage and death.

Apoptosis could occur during cryopreservation as a result of ice formation and/or CPAs toxication leading to cell death. During cryopreservation using slow freezing methods, cryoprotectants penetrate cell and reduce the ice formation in the surrounding solution and protect cells from rupture and death by replacing cytoplasm water with CPAs, however high concentration of CPAs could be lethal to cells, furthermore slow freezing can reduce cell damage while maintaining cell membrane permeability (Young et al., 2012).

Apoptosis and necrosis inhibitors such as Z-VAD, p38 MAPK inhibitor, ROCK inhibitor, and others are being included into cryopreservation and post-thawing protocols. These inhibitors target/inhibit various proteins involved in the cell death cascade, such as caspases, proteases, and kinases. Therefore, resulting in reduced apoptosis and necrosis in cells by supplementing cryopreservation solutions with these inhibitors would help in maintaining cell viability, thus improving the overall efficiency of cryopreservation. Also post-

cryopreservation mitochondrial-mediated apoptosis due to mitochondrial stress has been reported, therefore understanding the molecular mechanism of mitochondrial stress might enhance the cryopreservation outcome (Stéphenne et al., 2007).

Initial experiment conducted by Jitraruch et al. (2017), to cryobreserve HMBS, found that addition of anti-apoptosis compound ZVAD, an iron chelator such as deferoxamine (DFO), and human serum albumin (HSA) to the cryopreservation solution enhanced the cryopreservation outcome by protecting hepatocytes from oxidative stress and apoptosis, and helped in maintaining HMBs integrity (Jitraruch et al., 2017).

7.1.1. Apoptosis and Gene Expression

Recent studies showed that apoptosis is part of cryopreservation induced cell injury, therefore understanding the apoptosis pathway during cryopreservation could enhance the cryopreservation outcomes.

Apoptosis is regulated by two main mechanisms: extrinsic and intrinsic pathways. In the extrinsic pathway, apoptosis is caused by the death ligand, which activates the death receptor on the cell surface. The death receptor then attaches to the Fas-Associated Death Domain (FADD), which is found in the death-inducing signalling complex (DISC), which also contains caspase-8 and caspase-10, which later proceed to caspase 3, caspase 6, and caspase 7, which eventually leads to apoptosis. On the other hand, intrinsic pathway is triggered by activated caspase8. This causes alterations in the permeability of the mitochondrial outer membrane, resulting in the release of cytochrome c into the cytoplasm, followed by the activation of caspase 9, which results in cell death. Furthermore signals from external stimuli can promote DNA damage in another intracellular mechanism (Xu et al., 2010).

Apoptosis is caused by the activation of p53 in the intrinsic pathway. Normally, p53 expression is maintained at a level sufficient in the cytosol preventing it from entering the nucleus. When cell under stress, p53 attaches to the outer mitochondrial membrane, causing changes its permeability, which subsequently stimulates the release of cytochrome *c*, leading to caspase activation and cell death by apoptosis (Xu et al., 2010).

7.1.2. Caspase Activity

Caspase activity, is involved in the apoptotic pathways. Zhang et al. (2013) showed that cryopreservation of granulosa cells induced oxidative stress which resulted in the activation of caspase 8, caspase 9, and caspase 3 dependent apoptotic pathways (Zhang et al., 2013).

Several important proteins, including Bcl-2 family members, cytochrome *c*, and apoptosis protease activation fac- tor-1 (Apaf-1), are expressed at different levels at the time caspase activation is taking place apoptosis (Desoutter et al., 2019). In another study by Vining et al. (2021) in which they cryopreserved animal oocytes and embryos, they found that apoptotic factors including Bcl-2 and Bax gene expression changes in response to cryopreservation, according to comparisons between fresh and cryopreserved samples.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a pleiotropic enzyme that is overexpressed in apoptosis and several chronic diseases in humans. The protein triggers a cyclosporin A-inhibitable permeability transition that includes a loss of inner transmembrane potential, matrix swelling, inner mitochondrial membrane permeabilization, and the release of two pro-apoptotic proteins, cytochrome *c* and apoptosis-inducing factor (AIF). GAPDH's new role might have ramifications for mitochondrial biology, oncogenesis, and apoptosis research,

however, it is used as housekeeping gene for the normalization step of other genes in gene expression assays (Tarze et al., 2007).

The aim of this work was to study the effects of addition of anti-apoptotic agents, and other cryoprotectant(s) to the cryopreservation solutions on apoptotic genes expression in cryopreserved rat hepatocytes compared to fresh cells. cell.

7.2. Materials and methods

7.2.1. Equipment and materials

- NanoDrop (ND-1000 Spectrophotometer, Thermo fisher scientific, USA).
- QuantaStudio 5 Real Time PCR (Thermo fisher scientific, USA).
- 1.5 ml Eppendorf tubes.
- PCR Plates 384 well.

7.2.1.1. Hepatocyte Culture

Cryopreserved rat hepatocytes were thawed and cultured for 24, hours as described in Chapter 2, Section 2.2.3.

7.2.1.2. RNA Extraction

- Direct-zol™ RNA Mini prep extraction kit (ZAYMO Research, USA).
- TRIzol® Reaqent (Ambion, life technologies, UK).

7.2.1.3. cDNA

- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, USA).
- Qiagen Omniscript® RT Kit (Qiagen, Germany).
- Oligo(dT)$_{12-18}$ Primer (Invitrogen, USA).

7.2.1.4. RT-PCR

- TaqMan® Gene Expression Assays (Thermo fisher scientific, USA).
- TagMan™ Universal Master Mix II no NUG (Thermo fisher scientific, USA).

7.2.2. Methods

7.2.2.1. Experimental Design

Cryopreserved hepatocytes were thawed evaluated for viability then cultured for 24h at 37C incubator. Hepatocytes were harvested then RNA was extracted, and reversed transcribed into cDNA followed by RT-PCR using TaqMan® gene expression assays (Thermo Fisher Scientific). Five gene expression assays were selected and listed in **Table 7.1**, they included Bcl-2, Bax, Caspase 3, Caspase 9, and the housekeeping gene GAPDH. Data obtained from the real-time PCR machine were analysed and gene expression was presented as relative quantification.

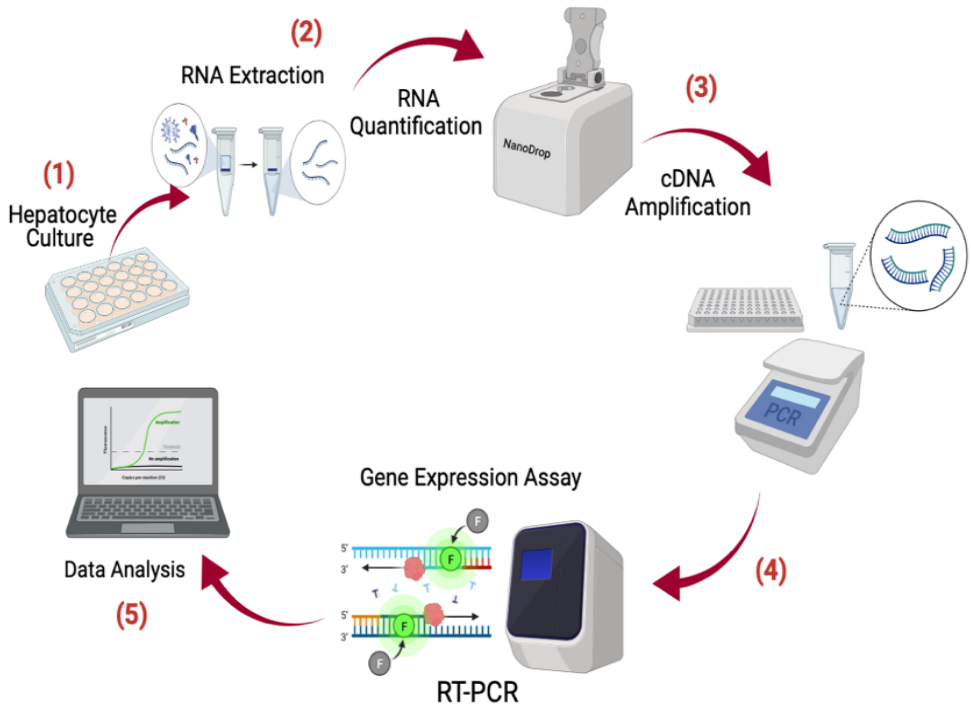


Figure 7.1: Experimental Designee for Apoptosis Gene Expression Assay

[Illustration created using BioRender platform, BioRender.com]

7.2.2.2. RNA Extraction and cDNA Amplification

Cryopreserved hepatocytes with different conditions that gave good cryopreservation outcome were selected as descried in **Table (7.1)**. hepatocytes were thawed and cultured for 24 hours followed by RNA extractions using Direct-zol^{TM} RNA Mini prep extraction kit as described in Chapter (2) Section (2.2.19). After that RNA was quantified by Nanodrop, then Reverse-transcription (RT) were conducted to produce cDNA using Qiagen Omniscript® RT Kit as described in Chapter (2) Section (2.2.21).

Table 7.1: UW solution with different concentration of cryoprotectants& Cryo-JIN

Cond 1	Cond 2	Cond 3	Cond 4	Cond 5
- UW - 10% DMSD - 5% glucose	- UW - 10% DMSD - 5% glucose - 60 μM Z-VAD	- UW - 10% DMSD - 0.2 M Trehalose - 60 μM Z-VAD	- Cell Banker	- Cryo-JIN

7.2.2.3. TaqMan® Gene expression Assays and Rea-Time PCR

cDNA samples of 20 µl each condition was used for real-time polymerase chain reaction to detect the expression of apoptosis genes (apoptosis: Bax, Bcl2, Casp3 and Casp9; housekeeping: GAPDH), qPCR cycle is shown in **Figure (7.2)**. qPCR run was conducted using TaqMan® Gene Expression Assays as described in Chapter 2, Section 2.2.21.

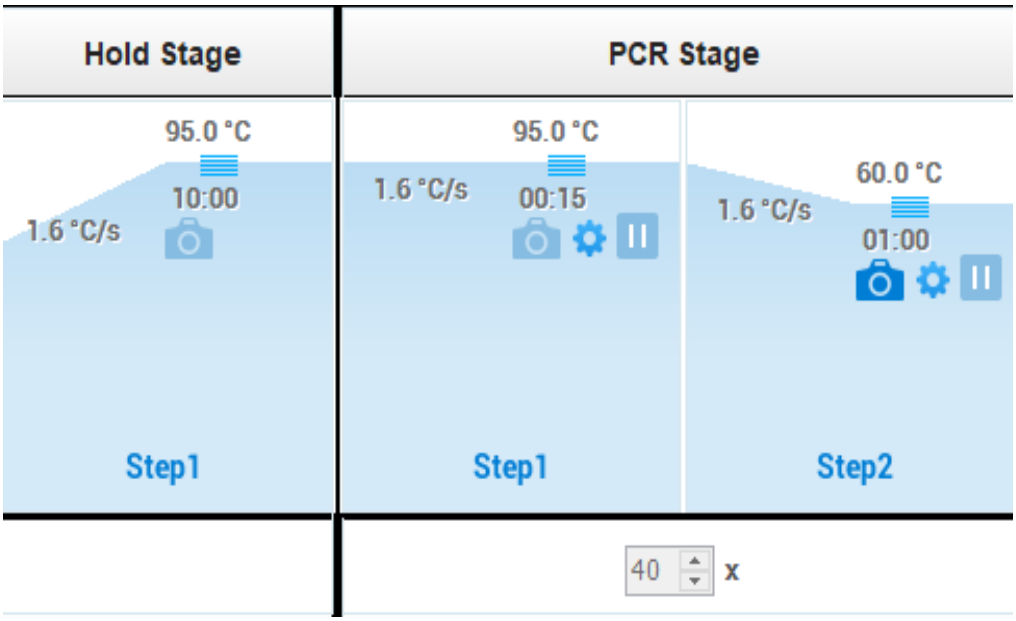


Figure 7:2: Gene Expression RT-PCR Program

Table 7.2: List of TaqMan® Gene Expression Assays				
Gene symbol	**function**	**Assay ID**	**Dye**	**Amplicon length**
Bax	Pro-apoptotic	Rn01480161_g1	FAM-MGB	63
Bcl2	Anti-apoptotic	Rn99999125_m1	FAM-MGB	104
Casp9	Pro-apoptotic	Rn00581212_m1	FAM-MGB	68
Casp3	Pro- apoptotic	Rn00563902_m1	FAM-MGB	93
GAPDH	Housekeeping	Rn01775763_g1	FAM-MGB	174

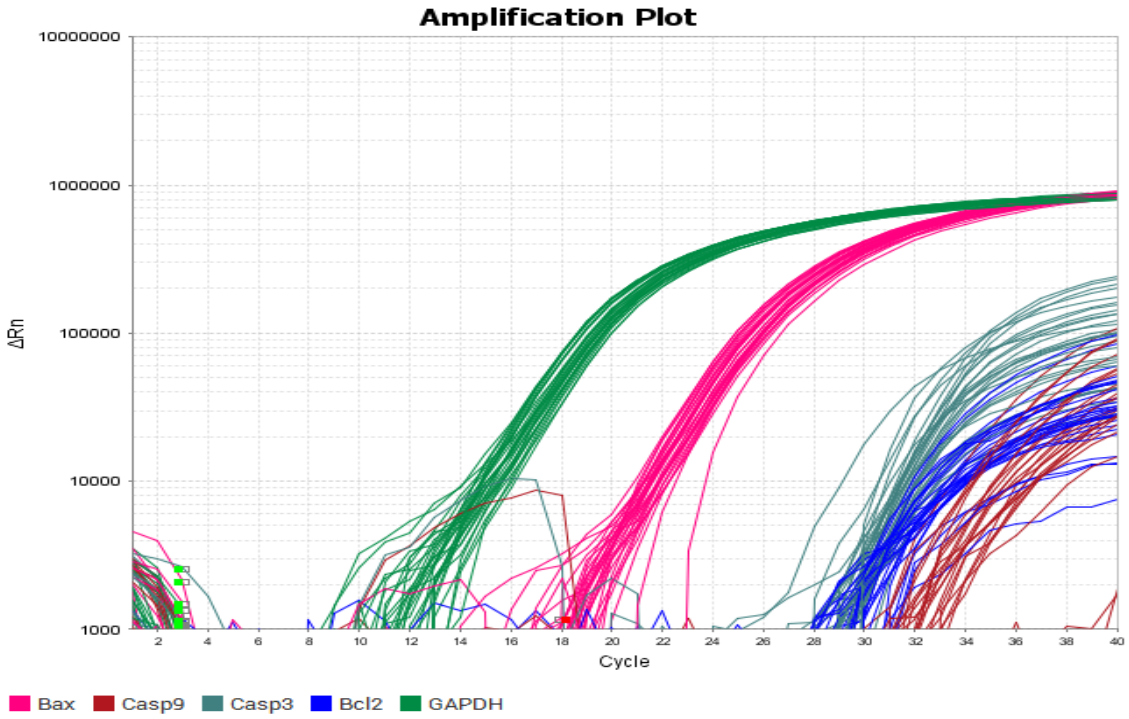
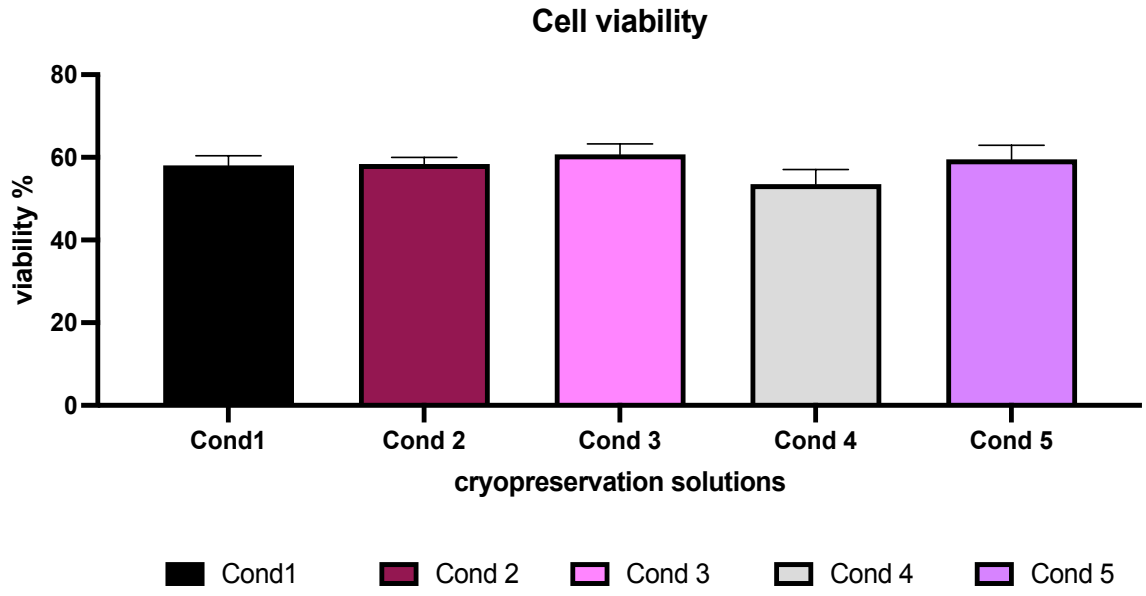


Figure 7.3: Gene Expression Amplification Plot

7.3. Results

Hepatocytes are very fragile and affected with different condition of extraction, cryopreservation, and post tawing, apoptosis could occur at any point of these processes. In clinical transplantation hepatocytes are thawed and checked for viability, then transplanted immediately into the patient. Earlier studies showed that cryopreservation could induce apoptosis in hepatocytes (Matsushita et al., 2003), this chapter aims to investigate the effect of cryopreservation process on hepatocytes viability and apoptosis on gene expression level, viability of thawed hepatocyte was determined using trypan blue exclusion and FDA/PI stanning **Figure (7.4)**.

7.3.1. Hepatocyte Viability post cryopreservation

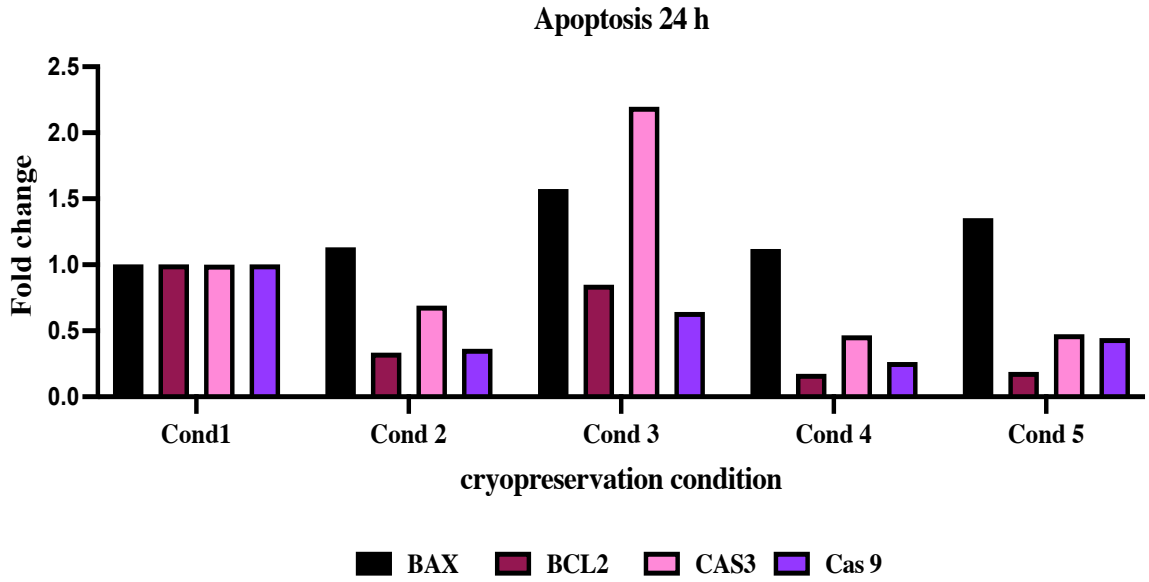


Cond 1	Cond 2	Cond 3	Cond 4	Cond 5
UW 10% DMSD 5% glucose	UW 10% DMSD 5% glucose 60 µM Z-VAD	UW 10% DMSD 0.2 M Trehalose 60 µM Z-VAD	Cell Banker	Cryo-JIN

Figure 7.4: Cryopreserved hepatocyte viability on thawing. Cells were cryopreserved in different cryopreservation solutions, and cell viability on thawing was checked using trypan blue exclusion test. Data showed that all cell viability were very similar.

7.3.2. Gene expression Assay

The results showed that the cryopreservation solution supplemented with Z-VAD and trehalose had higher expression of the anti-apoptosis gene BCL-2, which means that the addition of Z-VAD and trehalose to the cryopreservation solution supressed the apoptosis, were the other solution maintained similar level or higher level of the pro-apoptotic gene BAX, which means that the hepatocytes start getting into apoptosis.



Cond 1	Cond 2	Cond 3	Cond 4	Cond 5
UW 10% DMSD 5% glucose	UW 10% DMSD 5% glucose 60 µM Z-VAD	UW 10% DMSD 0.2 M Trehalose 60 µM Z-VAD	Cell Banker	Cryo-JIN

Figure 7.5: Apoptosis gene expression Assays**.** Relative quantification data are presented as fold change in gene expression compared to control. A fold change of ≥2 was considered as significant.

7.4. Discussion and Conclusion

Apoptosis could occur during cryopreservation as a result of ice formation and/or CPAs toxicity leading to cell death. During cryopreservation using slow freezing methods, CPAs penetrate cell and reduce the ice formation in the surrounding solution This would protect cells against death by the replacement of cytoplasm water with CPAs and preventing cell swelling and rupture, however high concentration of CPAs could be lethal to cells. Furthermore, slow freezing can reduce cell damage while maintaining cell membrane permeability (Young et al., 2012).

The relative quantification of apoptosis gene expression data **Figure (7.6)** showed that Bax (pro-apoptosis) expression was higher (1.1-1.5 folds) compared to control (cond 1). However, expression of Casp9 (pro-apoptosis) was significantly lower than that of control (0.2-0.5 fold change). Similarly, Casp3 (pro-apoptosis) expression was lower in cond 2 (0.55 fold change), significantly higher in cond 3 (2.2 folds change), and significantly lower in cond 4 and cond 5 (0.3 fold change in each) compared to control. On the other hand, Casp9 (pro-apoptosis) expression was significantly lower in cond 2 (0.25 fold change), in cond 4 (0.2 fold change), in cond 5 (0.2 fold change), and in cond 3 (0.75 fold change; was not significant) compared to control. Moreover, unexpectedly Bcl2 (pro-survival) expression was significantly lower than control in cond 2 (0.25 fold change), cond 4 (0.2 fold change), and cond 5 (0.2 fold change), while in cond 3 was not significantly lower (0.75 fold change).

It would be ideal if other cryoprotectant agents with better anti-apoptotic properties could be used to alter the pro-apoptosis/pro-survival gene expression balance to achieve enhanced cell survival.

In conclusion, although the use of the various cryopreservation solutions (5 conditions) resulted in similar cell viability on thawing, the apoptosis gene expression data showed that adding 60μM Z-VAD to UW solution had some effects on suppression of pro-apoptosis gene expression. However, cryopreservation solution UW supplemented with 60μM Z-VAD, 5% glucose, and 10% DMSO (cond1; King's protocol) gives better results suggesting that cells had a "better" protection against apoptosis compared to the other four conditions. Further studies are needed to investigate the use of other concentrations of Z-VAD and other cryoprotectant agents which have enhanced/better anti-apoptotic properties.

Chapter 8

General Discussion and Conclusions

8.1. Discussion

Hepatocyte transplantation is the promising alternative for liver orthotopic transplantation for the treatment of acute liver failure. Intraperitoneal transplantation of hepatocyte microbeads (HMBs) has been successfully established at King's College Hospital, London, for treatment of children with acute liver failure. The microbeads used were freshly produced at the time of transplantation. It would be ideal if these microbeads to be used are readily available, especially for emergency cases. This could only be achieved by establishing banked cryopreserved microbeads. Therefore, optimizing a protocol for the cryopreservation of hepatocyte microbeads is essential. Initial study of hepatocyte microbeads cryopreservation was carried out at King's (Jitraruch et al., 2014) using UW solution and controlled-rate freezer. The outcome of this study showed some encouraging results, however, further optimization of the protocol used was required. Therefore, this research project is mainly conducted to establish an optimized protocol for cryopreservation of HMBs.

In the first part of the project, isolation of hepatocytes from rats and unused human donor liver tissues successful. Later, encapsulation of hepatocytes procedures was carried out after optimizing the encapsulator settings in order to produce good quality microbeads. The optimization of the encapsulator settings using 250µm nozzle was successful as the microbeads were of good size (microbead diameter: mean 514.5 ± SD 54.55 µm) and morphology. These settings were: Frequency =1300Hz, Voltage =0.80kV, Amplitude = 4, and syringe pump speed = 300. The average size of microbeads and the above-mentioned settings was.

After optimizing the encapsulator settings, the next goal was to test some commercially available cryopreservation solutions for hepatocyte cryopreservation using controlled-rate

freezer (CRF). The first stage of the optimization was carried out by cryopreserving rat hepatocytes, using seven different ready-made cryopreservation solutions: TC-Protector, Cell Banker1, CP1, Cryo-JIN, NutriFreez, pZever and University of Wisconsin (UW) solution. The findings on hepatocyte viability after cryopreservation revealed considerable variations across the seven solutions tested. In comparison to the UW solution, Cryo-JIN, Cell Banker, and NutriFreez solutions showed similar cell viability after thawing. In comparison to other treatments, the viability of the TC-Protector, CP1, and pZever solutions was much lower. Cell viability on thawing data showed no significant differences between the tested solutions. However, the overall activity showed that hepatocytes cryopreserved in Cryo-JIN had a significantly higher overall activity compared to fresh and other cryopreserved cells. Moreover, both Cryo-JIN and Cell Banker 1 maintained a higher activity at all time points when compared to other solutions. A significant drop in overall activity with time was observed in all cryopreserved hepatocytes, however, Cryo-JIN and Cell Banker 1 maintained a higher activity compared to other solutions.

Fresh and cryopreserved hepatocytes were examined for cell attachment at 24, 48, and 72 hours, all solution showed reducing hepatocyte attachment with time points. Hepatocytes cryopreserved with Cryo-JIN, showed significantly higher levels of attachment than UW at baseline, while Cell Banker1, NutriFreez, and CP1 gave smeller results to UW solution. On the other hand, hepatocytes cryopreserved with TC-Protector and pZever solutions, showed significant drop-in activity.

Another hepatocyte-specific activity is urea synthesis, which is a measure of ammonia detoxification activity. Hepatocytes cryopreserved using Cryo-JIN produced higher urea levels compared to other cryopreservation solutions. Whereas TC-Protector and pZever had lower urea production compared to the other solutions. CP1, NutriFreez, and Cell Banker1 had similar urea production levels compared to UW solution.

Another important activity of hepatocytes is cytochrome P450 (CYP450) enzymatic activity, which is required for drug metabolism and toxins detoxification. The amount of induction of the xenobiotic-metabolizing enzyme cytochrome P-450 (CYP) 1A1/2 during the detoxification process by hepatocytes is measured using the ethoxy resorufin-O-demethylase (EROD) assay. The results showed that cryopreserved hepatocytes using UW, Ce1l Banker1, CP1, and NutriFreez solutions were all able to detoxify the xenobiotic agent through the activation of CYP 450 metabolic enzymes at similar levels; however, the activity of hepatocyte cryopreserved using TC-Protector and pZever was significantly lower than the other solutions and decreased over time.

Albumin synthesis by hepatocytes plays a primary role in the body, therefore maintaining albumin synthesis after cryopreservation is very crucial. All fresh and cryopreserved hepatocytes were able to synthesise albumin, which decreased over time; nonetheless, all solutions produced much less albumin, with the exception of Cryo-JIN, which produced more albumin than the other solutions.

After testing the seven ready-made cryopreservation solutions, additional optimization was done on the UW solution, because the initial data showed that (UW + 5% glucose + 10% DMSO) produced good cryopreservation results. Eight distinct cryopreservation conditions were prepared with UW solution every condition was assess for its efficiency for hepatocyte cryopreservation, each including different kinds and concentrations of cryoprotectants (CPAs) based on earlier published data; 0.2M Trehalose, 60 μM Z-VAD, 2% PVP, 1.5 M ETG. Hepatocytes were cryopreserved in each of the eight conditions and then compared to condition 1: UW + 5% glucose + 10% DMSO. Results showed that the addition of Z-VAD alone and/or Trehalose to UW solution enhanced the cryopreservation outcome.

Optimization of cryopreservation protocol for HMBs is the main goal of this project, enabling such protocol will assist the biobanking of HMBs for future clinical needs. Two

methods of cryopreservation were tested for HMBs cryopreservation, first the ***slow freezing methods*** using CRF was tested, then ***vitrification method*** was tested using liquid nitrogen.

After testing different types of cryopreservation solutions on hepatocytes, the same readymade solutions were then tested for HMBs cryopreservation. The seven-solution showed that HMBs overall activity, Cytochrome P450 (CYP450) activity, Albumin synthesis, and urea production when using UW, Cryo-JIN and Cell Banker1 gave similar levels compared to UW, however HMBs cryopreserved with TC-Protector, CP1, NutriFreez and pZever solutions showed significantly low activity compared to other solution. Therefore, the data suggested that these four solutions are not recommended for HMBs cryopreservation. On the other hand, in most of the experiments, Cryo-JIN maintained slightly higher levels of activities compared to the other solutions. Therefore, Cryo-JIN seems to be "best" for HMBs cryopreservation.

Based on the earlier results of hepatocyte cryopreservation using UW solution with addition of Z-VAD and Trehalose, which showed that this modified solution was suitable for hepatocytes cryopreservation, therefore, it was decided to use it for HMBs cryopreservation. The HMBs were cryopreserved in UW solution supplemented with 10%DMSO, 1.5% M ETG, 0.2M Trehalose, and 60 μM Z-VAD, and compared to UW with added 5% glucose, and10%DMSO,. Moreover, Cryo-JIN was tested too. The results showed that both WU/DMSO/Glucose and Cryo-JIN solutions gave similar cryopreservation outcomes, however UW solution supplemented with Z-VAD, and Trehalose resulted in slightly higher activities.

In addition to ***slow cryopreservation method*** using the CRF, HMBs cryopreservation was tested using the ***vitrification method***. FDA/PI viability stanning showed that HMBs were viable post-vitrification, but unfortunately some cracks were seen in some microbeads in the all tested solutions, which is unfavorable for the transplantation purpose, therefore HMBs vitrification has failed.

Apoptosis and necrosis are the main challenges facing cryo-biologists. Developing cryopreservation protocols and studying the effect of cryoprotectants on cells during freezing process and thawing helped in understanding mechanisms of ice formation and preventing cell death during and post cryopreservation. To study the beneficial effects of adding CAPs and anti-apoptotic agent(s) to the cryopreservation solution, four apoptosis genes were in rat hepatocytes: Bcl-2 (pro-survival), and three pro-apoptosis genes Bax, Casp-3, and Casp-9. Cryopreserved hepatocytes were thawed and cultured for 24h, then RNA was extracted, and reverse transcribed into cDNA and used in gene expression assays. The obtained data suggested that adding Z-VAD to UW solution enhanced the cryopreservation outcome by suppressing apoptosis pathway, however, further optimisation using Z-VAD at different concentration(s) could further suppress apoptosis.

8.1.2. Limitations of the Study

Limited availability of "good" quality human donor liver tissue (Mitry et al., 2003), If time permitted, other cryoprotectants of interest could be tested such as; mitochondrial stress, and oxygen consumption could be studied to detect the effect of the cryoprotectant used for cryopreservation, such as using Seahorse® or Oroboros®.

8.2. Conclusions and Future Work

8.2.1. Conclusions

In conclusion, an optimised protocol for cryopreservation of hepatocyte microbeads was achieved by using slow freezing method using CRF, were readymade cryopreservation solutions Cell Banker1, and Cryo-JIN resulted in improved hepatocyte functions and activity compared to control. Moreover, University of Wisconsin solution supplemented with DMSO, trehalose and Z-VAD enhance hepatocyte, and hepatocyte microbeads cryopreservation outcome compared to control. On the other hand, hepatocytes microbeads cryopreservation using vitrification, resulted with cracked and broken HMBs, therefore vitrification with the tested solution is not recommended for HMBs cryopreservation.

Finally, hepatocytes and HMBs could be cryopreserved using the slow freezing method using Cell Banker1, and Cryo-JIN readymade commercially available solution and by using the modified protocol UW solution with the addition of DMSO, trehalose and Z-VAD.

8.2.2. Future Work

If time permitted there are some other cryoprotectants that would of interest to test on cryopreservation of RMBs first, then HMBs. For example, wheat proteins which have been proven to improve the outcome of rat hepatocytes cryopreservation. Also, 1K1 which is an "engineered" form of hepatocyte growth factor (HGF) and works as caspase inhibitor and was shown to improve cell viability on thawing.

Furthermore, oxidative stress is often implicated as one of the injury mechanisms during cold storage both with and without ice formation. Production of reactive oxygen species (ROS) and lipid peroxidation are considered as indicators of oxidative damage. Therefore, studying ROS post thawing would be of interest.

Transplantation of microbeads in ALF animal model, will give us more indication on how RMBs could support the failing liver and survival, and would be compared with fresh RMBs.

Co-encapsulation of hepatocytes with mesenchymal stromal cells (MSCs) is another research idea, as it has been shown that co-transplantation of hepatocytes and MSCs lead to more beneficial outcome than single-cell transplantation in the treatment of ALF (Kong et al., 2020). They demonstrated that HNF4α-overexpressing human umbilical cord MSCs (HNF4α-UMSCs) enhanced the expression of hepatic-specific genes. In alginate–poly-L-lysine–alginate (APA) microbeads, HNF4-UMSCs improved the function of primary hepatocytes, and when transplanted in an ALF mice model, superior therapeutic benefits were obtained

Moving from HMBS to 3D printing of hepatocyte/alginate could be tested in BAL systems.

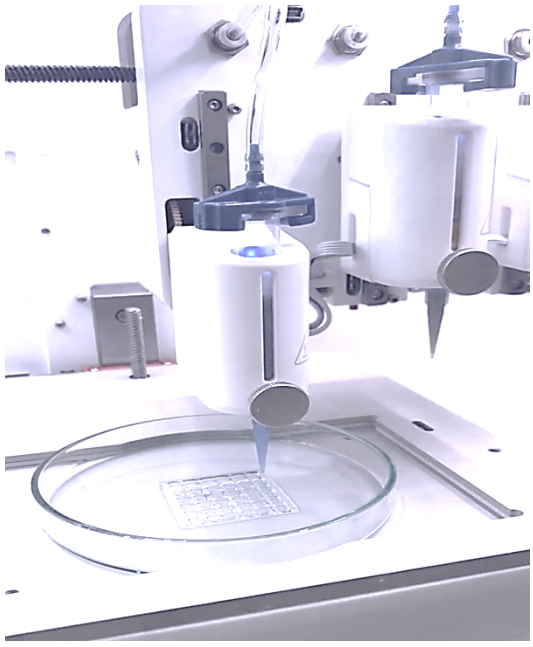


Figure 8.1: Initial experiment of 3D printing of hepatocyte in SLG20 Alginate [Almazrouei unpublished data]

Finally, HMBs with depolymerised centre might lead to the formation of hepatocyte spheroids as a result of cell-cell direct contact leading to enhanced hepatocyte-specific function and activity. Furthermore, if these HMBs could be also cryopreserved and banked for emergency use.

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Appendix I

List of Abstracts and Publications

Abstracts

Almazrouei S, (2018). Oral Presentation of the work plan of PhD study: "Optimization of Hepatocyte Microbeads Cryopreservation Protocol". ***Institute of Liver Studies Research Meeting***, May 2018, James Black Centre, King's College London, Denmark Hill Campus.

Almazrouei S, Dhawan A, Filippi C, and Mitry RR, (2019). Poster Presentation of the work entitled: "Establishing an optimized protocol for cryopreservation of hepatocyte microbeads for clinical use". The first ***SIMS Postgraduate Symposium***, 6th September 2019, King's College London - the Strand Campus.

Almazrouei S, (2020). Oral Presentation of the work entitled: "Hepatocyte Microbeads Transplantation". ***SKC Educational Program***, SEHA, 15th September 2020, Abu Dhabi, United Arab Emirates.

Almazrouei S, (2022). Abstract submitted "Cryopreservation of Hepatocyte Microbeads for Clinical Use", ***CRYO2022*** - 59th Annual Meeting of The Society for Cryobiology, 9-22 July 2022, Dublin, Ireland.

Publications

"Hepatocyte Cryopreservation Using Readymade Solutions".
[manuscript in preparation]

"Optimization of Hepatocyte Microbeads Cryopreservation Protocol".
[manuscript in preparation]

Appendix II

Human Hepatocyte used for experiments			
Batch Number	**Viability**	**Cell Count**	**Experiments**
HC 365	73%	14.75X107	Fresh
HC379	75%	32.74X107	CRF Hep + HMB Cryo
HC380	74%	21X107	CRF Hep + HMBs+ LN Cryo
HC381	82%	12.54X107	CRF HMBs Cryo
HC387	85%	597.8X107	CRF HMB+ Hep Cryo
HC388	87%	64.6X107	HMBs LN Cryo
HC389	78%	35.6X107	HMBs LN Cryo
HC390	75%	33.12X107	CRF Hep Cryo
HC391	92%	15.19X106	CRF Hep Cryo
HC394	75%	48.8X106	CRF Hep + HMBs Cryo
HC395	82%	73.8X106	CRF Hep Cryo
HC397	85%	41.5X106	CRF HMB+LN Cryo
HC400	84%	130X106	CRF HMB+ LN Cryo
HC404	65%	121X106	CRF HMB + LN Cryo
HC405	65%	100X106	CRF HMB + LN Cryo
HC406	73%	24X107	CRF HMB + LN Cryo
HC407	77%	98X107	CRF HMB + LN Cryo
HC410	74%	14.4X106	CRF Hep Cryo
HC412	89%	120X107	CRF Hep Cryo

Appendix III

Rat Hepatocyte Used for Experiments			
Batch Number	**Viability**	**Cell Count**	**Experiment**
RH 11	70%	1.86X107	Fresh
RH 12	75%	30.3X187	Fresh
RH 13	78%	12.5X107	Fresh
RH 14	73%	23.2X107	CRF RHC Cryo
RH 18	78%	40.5X107	CRF RHC Cryo
RH 19	77%	27.3X107	CRF RHC Cryo
RH 20	74%	52.3X107	CRF RHC Cryo
RH 21	80%	37.0X107	CRF RHC Cryo
RH 22	74%	80.0X107	CRF RHC Cryo
RH 23	77%	34.7X107	CRF RHC Cryo
RH 24	60%	15.5X107	CRF RHC Cryo
RH 28	76%	26.8X107	CRF RHC + RMB + LN Cryo
RH 29	77%	45.5X107	CRF RHC + RMB + LN Cryo
RH 30	86%	36.7X107	CRF RHC +RMB + LN Cryo
RH 32	70%	13.90X107	CRF RHC Cryo
RH 33	75%	46.4X107	CRF RHC Cryo
RH 34	80%	15.2X107	CRF RHC Cryo
RH 35	76%	97.2X107	CRF RHC + RMB + LN Cryo
RH 36	83%	43.2X107	CRF RHC+ RMB+ LN Cryo
RH 37	78%	42.9X107	CRF RMB+ LN Cryo
RH 38	81%	40.0X107	CRF RMB+ LN Cryo