BIOPRESERVATION OF HEPATOCYTES: CURRENT CONCEPTS ON HYPOTHERMIC PRESERVATION, CRYOPRESERVATION, AND VITRIFICATION

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Abstract

Isolated liver cells (primarily isolated hepatocytes) have found important applications in science and medicine over the past 40 years in a wide range of areas, including physiological studies, investigations on liver metabolism, organ preservation and drug de-toxification, experimental and clinical transplantation. An integral component of many of these works is the need to store the isolated cells, either for short or long-term periods. This review covers the biopreservation of liver cells, with a focus on the history of liver cell biopreservation, the application of hypothermia for short-term storage, standard cryopreservation methods for isolated hepatocytes, the biopreservation of other types of liver cells, and recent developments such as vitrification of hepatocytes. By understanding the basis for the different approaches, it will be possible to select the best options for liver cell biopreservation in different applications, and identify ways to improve preservation protocols for the future.

Keywords: Isolated hepatocytes; isolated liver cells; biopreservation; hypothermic storage; cryopreservation; vitrification; sub-zero non-freezing storage; drying.

INTRODUCTION

It is more than 40 years since the definitive studies of Berry & Friend [6] and Seglen [109] demonstrated that is was possible to isolate individual functioning hepatocytes from intact rodent livers by enzymatic digestion. This was a major step forward in facilitating research on biochemical and molecular aspects of liver function and pathology. It became possible to disaggregate a complex organ, such as the liver into cellular components, whilst keeping the functionality of its cells. An idea of the structural complexity of the liver is

appreciated in Figure 1. It became possible to isolate not only the hepatocytes (as the major fraction of metabolically-active cells) but also other cell types such as sinusoidal endothelial cells or Kupffer cells; however these are beyond the scope of this review.

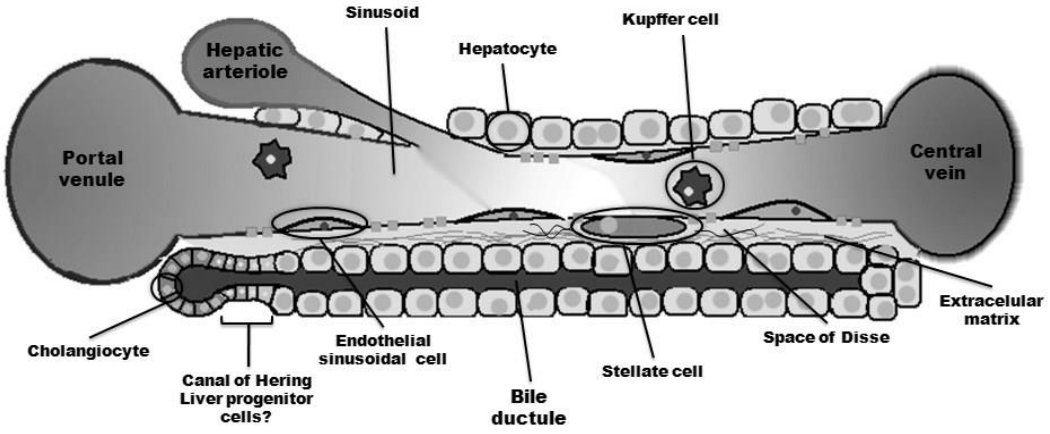

Figure 1. Liver cell organization. Dual blood supply of the liver merges upon entry into the liver lobule at the portal field. The four major cell types that are found in the liver are hepatocytes, stellate cells, sinusoidal endothelial cells and Kupffer cells.

The isolation technique has been subjected to various refinements to become a robust and easily-reproducible method between laboratories. Hepatocytes have been isolated from a growing number of species including several mammalian species in addition to rodents (such as dog [69], cat [111], rabbit [24], pig [11], goat [129], sheep [89], and also man [20, 88], and the technique has been applied in other phyla including fish [103], amphibians [10], archosaurs [45], and birds [43]. The uses of isolated hepatocytes have expanded over that time as greater understanding of their potential benefit has been accrued, and now include widespread use in metabolic studies, drug discovery, and experimental and clinical cell transplantation [122].

Mature hepatocytes are terminally-differentiated epithelial cells which in normal physiology possess a wide range of important functions, such as metabolic conversion and detoxification of xenobiotics, control of intermediary metabolism, interconversion of sugars, lipids and amino acids, and synthesis of proteins. Whilst mammalian hepatocytes can be successfully sustained in tissue culture for a few days, they do not undergo replication to any significant degree, even when stimulated with potent growth factors. The high level of interest in using cells and the fact that liver tissues can yield high numbers of cells from one isolation (e.g. for human livers, $>2x10^6$ cells per g liver weight [125] have led to an associated need to preserve the isolated cells to maximise the benefit from isolation, bank large cell numbers for transplantation or provide good inter-batch comparisons for xenobiotic assays. Over the same time period, there has been an increasing interest in studying other liver-derived cells, such as hepatoblastoma cells and liver progenitor cells, often as a way to derive continuously-replicating cells which possess some of the functional characteristics of mature hepatocytes, and which also require biopreservation for similar reasons.

Biopreservation methods have been almost exclusively based on applications of low temperatures, either hypothermic storage in the range of 2-4^{o}C, or cryopreservation to deep

subzero temperatures. In some cases, for specific purposes the hepatocytes require preservation in different formats such as encapsulated in spheroids of different biocompatible media, or in monolayers overlain with biopolymers.

This review will provide an overview of **1.** The historical background of liver cell preservation in the early years up to 1990; **2.** Current concepts: hypothermic preservation methods appropriate for a few days; **3.** Current concepts :Cryopreservation methods based on slow cooling regimes; **4.** Biopreservation at cryogenic temperatures in altered formats such as cell encapsulation; **5.** Biopreservation of liver-derived cell lines and hepatic progenitor cells; **6.** Alternative approaches to hepatocyte preservation, including vitrification, subzero non-freezing storage or dry state preservation.

1. THE EARLY HISTORY OF LIVER CELL BIOPRESERVATION – PERIOD UP TO 1990

Early work into cryopreservation of isolated hepatocytes was reported by Le Cam and colleagues in the mid-1970's [59] using rat hepatocytes as the most easily available source. The chosen cryoprotectant (CPA) was dimethyl sulfoxide (Me_2SO) at concentrations up to 1.5 M, and exposure was carried out at 4^{o}C, indicating that even in the first experiments, issues with CPA toxicity were recognised. A range of cooling rates was investigated between -2°C/min and -100°C/min down to below -100°C as target temperature, before transfer to liquid nitrogen ($LiqN_2$). Thawing was done rapidly in a water bath at 37°C. The best recoveries were found when cooling rates were in the range of -2°C to -7°C/min, with viability by trypan blue (TB) staining at around 80%. Nevertheless, more complex metabolic mechanisms, assessed by gluconeogenesis, were compromised at around 50%.

The value of using a sugar (in this case, glucose) as an osmotic buffer during a slow cooling protocol for rat hepatocytes was shown by Fuller et al [37] when using Me_2SO as the preferred CPA. In experimental transplant studies from Kusano et al, [57] rat hepatocytes recovered after slow cooling / rapid warming using Me_2SO were shown to survive morphologically when grafted into the splenic pulp of recipients as isografts (a location for heterotopic hepatocyte transplantation which was originally reported by a number of groups at the time [93] and which remains a mainstay of the procedure until today).

Investigations into other CPA for rat hepatocytes confirmed that Me_2SO was superior to glycerol or methanol using slow cooling / rapid warming, and again the range between -1°C/min and -10°C/min was optimal as assessed by TB staining and urea synthesis. However, and once again, more complex metabolism (in this case, protein synthesis) remained low after recovery. One notable finding was that exposure to Me_2SO did not itself impact protein synthesis, as long as a sugar (in this case, glucose) was used as an osmotic buffer in the CPA dilution step [37].

Further studies were undertaken where rat hepatocytes during slow cooling with Me_2SO were rewarmed at various intermediate temperatures down to -60°C, the end target temperature, and even though high yields could be recovered by TB staining, ultrastructural analyses immediately on thawing showed many changes compared to the starting cell population [35]. These ranged from mild swelling of mitochondria and cytoplasmic vacuolation to condensation of organelles into the cell interior and blebbing of the plasma membranes.

Use of high cooling rates (approximately -300°C/min) resulted in high frequencies of severe abnormalities with extensive disruption of organelles and cytoplasm, possibly suggesting intracellular ice formation. Bilirubin conjugation could be measured in rat hepatocytes recovered after slow cooling, but was not detectable in these rapidly-cooled cells. In other work, microsomal drug detoxification was partially conserved in rodent hepatocytes

after slow cooling / rapid warming [92] and again, Me_2SO proved to be a better CPA than glycerol.

During this time, interest in transplanting cryopreserved hepatocytes for liver support, at least in experimental models, continued to grow, and infusions of cryopreserved rat hepatocytes were made to support essential detoxification processes in models of acute liver failure (produced either by surgical intervention or chemical poisoning). In a rodent model, cryopreserved hepatocyte isografts or xenografts (cryopreserved pig hepatocytes) infused into the peritoneal cavity both showed evidence of life-sustaining support [71]; however, the exact functional status of the cryopreseved cells at the point of infusion was unknown, and viable cells could not be detected in the peritoneum. Nevertheless, using intrasplenic transplantation in rodents, several groups were able to show long-term survival of at least a population of cells from cryopreserved hepatocytes [36, 68]. Rat hepatocytes cryopreserved using slow cooling and Me_2SO as CPA could be identified in the spleen at 6 weeks after grafting, and post-thaw recoveries could be evaluated by significant uptake of the radio-labelled scanning agent HIDA. Similar results were reported using slow cooling/ Me_2SO as CPA by another group [32].

Alongside the studies on rodent hepatocytes, gradual refinement of the cell isolation methods permitted good recoveries of functional cells from livers of larger animal species and human livers. Cryopreservation was equally important for the work, which in this era was focused mainly on hepatocytes as models in drug metabolism and toxicology. The standard accepted protocol continued to be slow cooling with Me_2SO and rapid warming, which provided human hepatocytes post-thaw with good recovery of ultrastructural morphology [87] and drug metabolising activities [64]. However, in most cases and for different drug metabolising pathways, the cryopreserved hepatocytes showed reduced functions when compared with freshly-isolated cells. Intracellular ice formation or exposure to hyperosmotic solutions remain the main issues of cryopreservation process, and their effects on cell quality and cell death induction must be clarified.

Throughout the same time span, some investigators performed studies on hepatocytes preserved by hypothermic storage, both for practical short-term preservation to meet laboratory demands, and as a tool to investigate the compositions of novel hypothermic preservation solutions for whole organ storage for transplantation. The advantages of using isolated liver cells, obtained in large numbers from one liver perfusion, include the ability to test several variables within one experiment, and to have an easily manipulable test system where drugs or additives can be directly delivered to the cells in known doses.

Isolated rodent hepatocytes were used by Umeshita and colleagues to investigate the role of calcium in the preservation solution for hypothermic storage (2°C) between 12-72h [128], and Kravchenko studied morphological characteristics [52]. Fox et al [31] in Belzer's laboratory studied the importance of pH control when storing hepatocytes in either organ preservation solution (UW solution) or tissue culture medium over the same time period. Belzer's group went on to further investigate the role of colloids such as polyethylene glycol [76] and amino acids in the supporting medium [74] during hypothermic storage. It was generally agreed from these early reports that cold preservation injury increased beyond 24h storage, and was significant by 72h.

2. CURRENT LIVER CELL BIOPRESERVATION - HYPOTHERMIC STORAGE

Considering the relative sensitivity of hepatocytes to cryogenic preservation (see Section 3), hypothermic storage of hepatocytes in suspension is still a method of choice in many

practical situations due to simplicity and ability to study basic principles of cellular metabolism under cold hypoxia. Isolated liver cells also became an essential part of the field of cryobiology that outlined competent methods for cell preservation and clinical application for treating various forms of liver failure [34, 44] over the past three decades. Nevertheless, hypothermic preservation imposes a considerable impact on isolated liver cells. It is further compounded by the fact that hypoxia in the sample may develop when the hypothermic cell suspensions are kept in tubes or vials without further oxygenation – hepatocytes, being large cells, will readily sediment by gravity into densely-packed agglomerates -. The major challenges are characterized by multiple biochemical and ultrastructural changes (see figure 2) which can be summarized as follows:

- Perturbation of ion balances caused by hypothermia, notably mono- and divalent cations (Na^+, K^+, Ca^{2+}) and anions (Cl^-).
- Osmotic swelling.
- Shift in extracellular and intracellular pH.
- Oxidative stress.

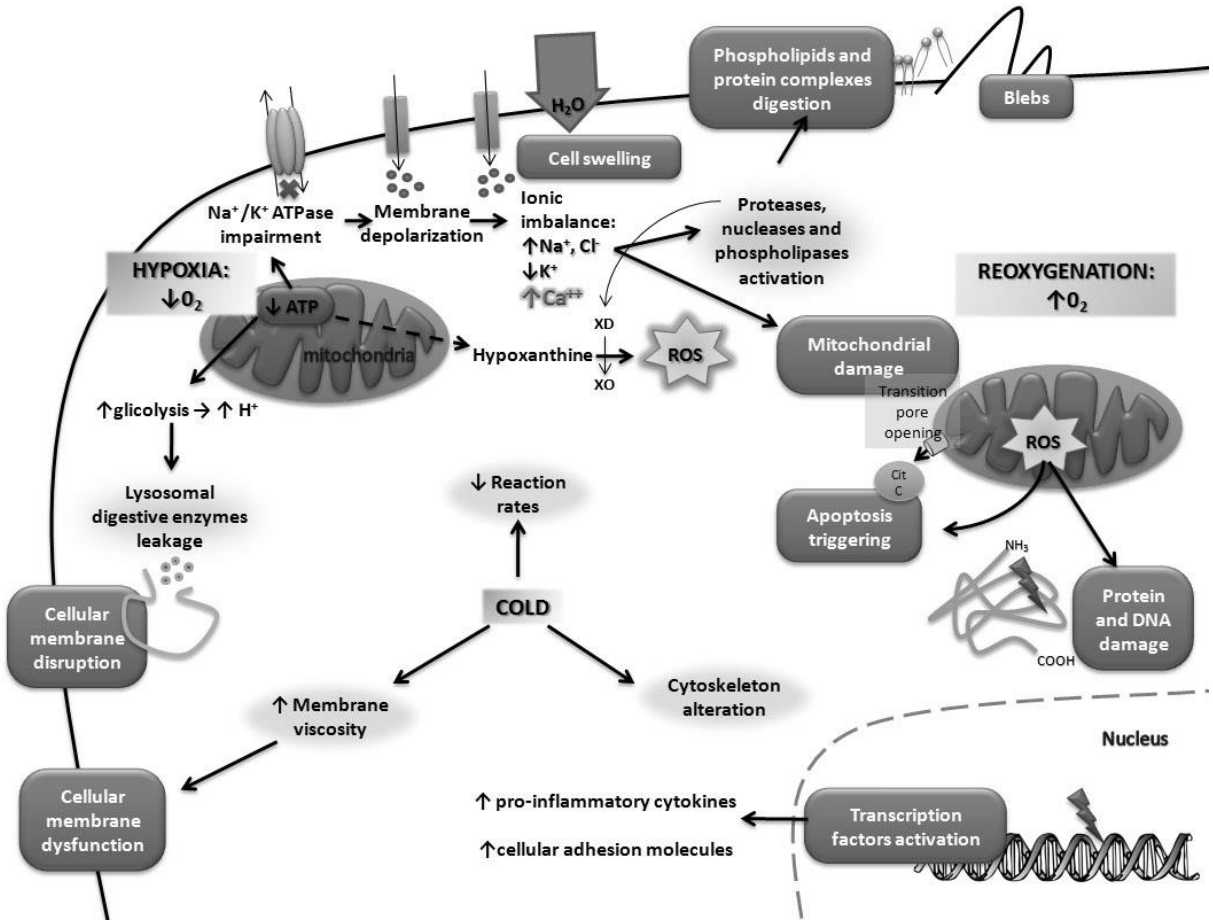

Figure 2. Hypoxia-reoxygenation phenomena in hepatocytes. Hypoxia, leading to fall in ATP and increase in H^+, affects membrane transporters and internal ion balances. Along with enzymes activation, cell and mitochondrial membrane injury occurs, leading to apoptosis and other molecular degradation. On re-oxygenation, injured mitochondria and ongoing enzymatic processes increase oxidative stress, and amplify the injuries via additional inflammatory processes.

a) Perturbation of ion balances.

Inhibition of the Na^+/K^+ ATPase causes cell edema, rapid depletion in ATP reserves, and a corresponding increase in ADP levels, and this depletion of ATP leads to the degradation of adenosine causing accumulation of hypoxanthine and xanthine oxidase. Cell membrane depolarisation also occurs very early in the cascade leading to a breakdown of ion homeostasis, and interplay of other intracellular and membrane - associated events that eventually culminate in cell death by either apoptosis or necrosis [41].

i) Perturbation of monovalent cations

Change in barrier function of liver cell membranes to monovalent cations and anions (K^+, Na^+, Cl^-, H^+) is well-recognized, leading to conditions of hepatocyte cellular water accumulation [15, 42]. Direct evidence showed that cooling of hepatocytes caused a K^+ loss and was accompanied by the intracellular diffusion of sodium and chlorine with associated volume increase [7].

ii) Calcium perturbation caused by hypothermia

Calcium plays a crucial role in biological systems. It is a universal cellular regulator of cell-to-cell interactions and mechanisms implemented to change cellular metabolism according to environmental conditions such as hypothermia. Loss in barrier function of cell membranes, particularly for bivalent cations, ensures a more severe injury of membranes and has a significant impact on hepatocyte function [29, 42, 128]. Cooling and changes in cell volume are drastic factors that mobilize calcium from intracellular depots [97]. Even short cold exposure of hepatocytes (20 min) in UW solution may cause a significant loss of intracellular Ca^{2+}. The main intracellular Ca^{2+} depots are endoplasmic reticulum and mitochondria which appear to be susceptible to hypothermia and promote rapid Ca^{2+} release [48]. Concomitant pretreatment with calcium blockers in the relevant intracellular compartments may stimulate ATP regeneration and improve hepatocyte cellular hypothermic preservation [28].

The optimal concentration of calcium in cellular environment may be a crucial factor for cell viability. Umeshita et al [128] suggested that optimal Ca^{2+} concentrations of the preservation solutions were within narrow range 0.3-0.5 mM. Addition of combinations of electrolytes of Ca^{2+}/ Mg^{2+} to the L-15 preservation medium suppressed hepatocyte swelling, increased endogenous respiration and improved viability during 48-hours cold preservation [74]. The possible protective mechanism of calcium may be attributed to cytoskeleton stabilization, followed by inhibition of bleb formation on plasma membranes and a decrease in LDH release [48]. The problem of Ca^{2+} concentration in the storage medium is often discussed in relation to UW solution. Lactobionic acid, which is a component of the solution, and similar to phosphate, can chelate calcium ions [46] hence substantially influence the divalent cation distribution between the hypothermic preservation solution and cytoplasm.

b) Counteracting osmotic swelling.

Swelling was one of the earliest changes registered in cold-preserved hepatocytes [7, 42, 76]. Many papers have recognized a link between alterations in ion balances, progressive edema and the loss of cellular viability [34, 75, 128, 130].

A major advancement in cold preservation has been made by including cell-impermeant osmotically active molecules to prevent the cold-induced swelling. Low-molecular weight substances such as glucose and galactose have shown little effect against edema. However, mannitol, lactobionate and sucrose, substantially improved hepatocyte integrity and can be considered to be the most popular choices as additives in hypothermic preservation.

Polymeric substances with osmotic and oncotic properties such as polyethylene glycols (PEGs) of different molecular mass (from 8 to 35 kDa) were shown to prevent effectively liver cell swelling, reduce LDH release and increase viability [73]. Similar substitution of PEG by dextran (5%) or saccharides (100 mM) neither prevented cell swelling nor protected the cells from death [76]. Based on personal experiences we noticed that, due to some sort of artifact, isolated cells in presence of PEG become refractive to trypan blue. For unexplained reasons, the viability assessed by trypan blue exclusion after cold storage, appears to be generally above 95% when trypan blue is assessed in media which contain residual PEG, irrespective of the treatment or real quality of cells. This is probably due to a synergistic effect between the interactions of the hepatocyte plasma membrane, the PEG, the low temperatures, and the trypan blue, reducing dye influx into injured cells during the analysis.

c) Significance of pH buffers during hepatocyte cold storage.

Maintenance of cell volume is closely related to cytosolic pH values via ion transport systems and exchangers such as Na^+/ H^+, Cl^-/ HCO_3^- and the Na^+/HCO_3^- symport which influences intracellular pH [83, 91]. In combination with low temperature, pH deviations may destabilize lysosomes and promote release of dehydrogenases in the mitochondrial matrix, with associated initiation of apoptosis [113]; and the situation becomes even more complex after rewarming. A return to normothermia disturbs regulatory mechanisms by attenuating Na^+/H^+ exchange and increasing Na^+/HCO_3^- cotransport, whereas Cl^-/HCO_3^- exchange is not affected [91].

At onset of cold hypoxia the expected pH shift to low values as H^+ accumulate, is compensated to some degree by buffering capacity of the cytosol [26]. According to the 'alpha-stat' theory, regulation of cellular pH in poikilothermic or homoeothermic animals is dependent on the temperature-dependent ionization of the α-imidazole group of histidine of cytoplasmic proteins [102], which results in a natural and opposing apparent alkaline shift in pH [25]. Histidine and carnosine were found to effectively prevent acidosis due to penetration and distribution in liver tissues [19, 114]. Citrate as a buffer was shown to penetrate and even modulate substrate turnover during cold storage, enhancing late hepatic synthesis of succinate, with possible metabolic benefits [66, 115].

The significance of pH stabilization and prevention of acidosis by a strong buffer system is an often-debated topic. It is well-defined that intracellular acidification can mediate the protective effects of hypothermic preservation on metabolism by one or more intracellular stress-activated enzymes such as phospholipase A. Cyclosporine A administration, which amongst other actions, blocks cytochrome c release from mitochondria (but not the rise of intracellular pH), significantly improved hepatocyte viability after reperfusion, suggesting the pivotal role of mitochondria in this process [101].

d) The role of antioxidant defense during cold storage.

Despite the fact that the end product of the electron transport through the mitochondrial respiratory chain is water, which is formed by the 4-electron reduction of molecular oxygen by cytochrome oxidase, part of the oxygen reduction machinery may be involved in one-electron reduction reactions, forming free radicals and oxidants such as superoxide anion O_2^-, hydrogen peroxide (H_2O_2) and most reactive hydroxyl radical •OH [113]. *In vivo* only a small portion of oxygen (approximately 5%) processed via mitochondrial respiration, is spent on the formation of O_2^- [113], but hypothermia and hypoxia lead to a significant increase of superoxide anion formation. Together with available data on significant production of reactive oxygen species (ROS) by mitochondria and their role in oxidative damage, Ohkohchi et al, demonstrated that the concentration of O_2^- in intact mitochondria is 2-3 thousand times lower than in sub mitochondrial particles. Therefore, only minor amounts of O_2^- "escape"

from the mitochondria, which is potentially damaging to their own stability [96], and providing antioxidants to the preserving solutions during hypothermic storage of liver cells is one feasible way to increase the overall antioxidant defense.

The relative high resistance of hepatocytes to oxidative stress, compared with endothelial cells, may be attributed to a high antioxidant capacity of hepatocyte cytoplasm. For instance, the concentration of reduced glutathione (GSH) in the intercellular space is around 10 μM, whereas its cytoplasmic concentration is more than 1000 times higher [58]. Although high levels of GSH can be present in native cells, hypothermia causes rapid depletion of the tripeptide [131]. It has been found that GSH content in whole liver after 5-6h cold storage dropped by 51%, and then by another 23% after subsequent reperfusion [134]. It is assumed that during hypothermia, hepatocyte plasma membranes become permeable to GSH [105]. Administration of exogenous GSH or methionine and their combination substantially slowed the GSH loss and protected viability during rewarming of cold-preserved hepatocytes [72]. The effectiveness of other antioxidants, such as those of plant origin, has been demonstrated in randomized studies. Silibinin from *Silybum marianum* added to preservation solution was beneficial for ATP content, reduction of lipid peroxidation, mitochondrial function and hepatocyte survival after storage at 4 degrees C. [54]. A water-soluble analogue of α-tocopherol provided antioxidant action during prolonged hepatocyte cold preservation up to 72 hours. This was accompanied by membrane stabilization thorough cytoskeleton proteins [48].

Many studies during the past decade have suggested a leading role for xanthine oxidase (XO), a powerful source of H_2O_2 and O_2 in hypothermic injury. XO inhibitors, such as allopurinol, demonstrated good scavenging ability during reperfusion of whole liver after hypothermic storage [47, 91]. It is important to note that inhibitors of XO, including allopurinol, are not totally effective, so the enzyme may still accomplish the electron transfer to oxygen thus generating superoxide [83]. Other XO inhibitors such as 4-Amino-6-hydroxypyrazolo pyrimidine (AHPP) lack such a propensity for radical leakage however, they may be associated with other toxicities which have prohibited wider clinical application. Although it is uncertain whether this finding is important for hypothermic storage of liver cells, it is important to realise that pharmacological properties of any drug may be different depending on temperature, different species or type of preservation solution. Development in the future of XO inhibitors which do not have a pro-oxidant action, similar to allopurinol, with associated minor side effects, would be an advantage.

3. CURRENT LIVER CELL BIOPRESERVATION: CRYOPRESERVATION OF CELL SUSPENSIONS

It has become a very essential problem to develop relatively simple and reliable methods for cryopreservation of isolated liver cells for long-term cryo-banking across a range of the applications discussed above. Despite the large number of papers devoted to this subject, the task of maintaining high levels of metabolic status in cryopreserved hepatocytes is still largely unresolved.

It must be remembered that isolated primary hepatocytes seem to be very sensitive to a number of factors associated with preservation, which include the important variables of the sources of the cells, and the methods used for their isolation. The initial state of the isolated hepatocytes may play a significant role in their responses to cryopreservation procedures. The donor species and size of the liver segment used for cell isolation, the original technique of liver procurement and its state (normal or pathological physiology, fat content, etc.) as well as duration of warm and cold ischemia; all have influences on the yields and viabilities of the isolated cells, most commonly produced by the enzymatic digestion method. However, for

hepatocyte isolation, it should be pointed out that there are two approaches for liver disaggregation. The most common approach includes two-step collagenase perfusion protocol [109], which disintegrates the extracellular collagen matrix. The final suspension of hepatocytes is obtained after mechanical dissociation, filtration and low speed centrifugation. Another different approach for hepatic cell isolation [53] allows an easy release of hepatocytes from small animal livers by a combination of liver perfusion at 37°C with calcium chelator, EDTA, followed by soft tissue disaggregation. The conditions during isolation must be carefully controlled; otherwise, problems of latent injury may arise in cells with damaged or altered cell membranes [121] which may impact in a negative way on subsequent cryopreservation.

For cryopreservation, the various important variables can be divided into four main categories: a. cryopreservation base medium; b. CPA type and conditions of addition and removal; and c. Cooling and warming profiles.

a) Cryopreservation base media

Commonly, the base of cryoprotective medium composes isotonic salt solution in extracellular concentration. Correspondingly, culture media such as RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), etc. are widely used as base solutions for cryoprotectants.

Later, solutions of "intracellular type" initially developed for hypothermic storage of organs for transplantation, were applied for hepatocyte cryopreservation. The "gold standard" of these solutions is University of Wisconsin (UW) solution, which contains the impermeable anion lactobionate (100 mM) and the impermeable saccharide raffinose (30 mM) [56]. Comparison of UW solution with three other solutions (DMEM, Cell Banker 1 and Cell Banker 2) for cryopreservation of hepatocytes with 12% Me_2SO and 10% fetal bovine serum demonstrated beneficial effect of UW solution on viability, enzyme release and plating efficiency of rat hepatocytes [5].

Application of UW solution is limited by rather high cost; therefore other solutions of intracellular-type were tested for hepatocyte cryopreservation. Sucrose-based solution allowed high post-thawing levels of rat hepatocyte viability, energetic parameters, cationic homeostasis [98] and O-demethylation of p-nitroanisole (CYT P450 activity) [81].

A recently developed dextran-based intracellular-type freezing solution, HypoThermosol (HTS), showed benefits when compared to DMEM in viability rate, albumin secretion, urea synthesis, deethylation of ethoxyresorufin (CYT P450 activity), and responsiveness to stimulation with interleukin-6 after rat hepatocyte cryopreservation with 10% Me_2SO [118]. However, data comparing UW to other intracellular-type solutions are unavailable in the literature.

b) CPA type and conditions of addition

Since hepatocytes are very cryolabile cells and their freeze-thawing without CPA results in the death of all cells in suspension a penetrative CPA such as Me_2SO, glycerol, propanediol, etc. is required. In most studies, Me_2SO demonstrated better recovery than propyleneglycol [100], propanediol and glycerol [14, 37]. In the study performed by Mazur et al, viability rate and detoxification activity of hepatocytes after cryopreservation with 1.4 M Me_2SO, glycerol, dimethylacetamide (DMAC) and 1,2-propanediol were compared [81]. Me_2SO and glycerol had higher cryoprotective effects than DMAC and 1,2-propanediol. Use of Me_2SO and glycerol showed similar high ability to preserve biotransformation of p-nitroanisole in isolated hepatocytes after cryopreservation.

The optimal concentration of Me_2SO in the cryopreservation medium comprises 1.25-2.5 M [14, 100], though usually its final concentration is 1.4 M, because higher concentrations are toxic.

Non-penetrative CPAs singly added to cryopreservation medium seem to not show a protective effect. However, their presence along with Me_2SO can increase cell survival and/or reduce the concentration of penetrating cryoprotectant. Thus, in a cryoprotective medium with Me_2SO, the addition of di-, tri-, and tetra-saccharides enhanced: viability, attachment to plastic and metabolic activity of rat and human hepatocytes in culture [85]. Among saccharides the most promising seems to be natural disaccharide trehalose, which demonstrated positive effects on the quality of not only several cell types, such as sperm [132], human oocytes [29], and erythrocytes [65], but also engineered tissue substitutes [13]. Supplementation of standard cryopreservation medium containing 10% Me_2SO with trehalose significantly improved post-thaw primary human hepatocyte viability, enzyme leakage, albumin and urea formation, cytochrome P 450 (CYP)-dependent xenobiotic metabolism, and plating efficiency. The useful property of trehalose to prevent cell death in cryopreservation probably is due to its ability to stabilize phospholipids and proteins during dehydration [27]. The inclusion of trehalose and related sugars (glucose, maltotriose) in the cryopreservation fluid in addition to dimethyl sulfoxide (10%), first tested with primary rat hepatocytes cooled in a controlled rate freezer, showed, after thawing, significantly higher viability based on TB of cells frozen in oligosaccharide-supplemented medium than for those cryopreserved without oligosaccharides [85].

The efficiency of supplementing the CPA base medium with high molecular weight polymers such as polyethylene glycols (PEG), to allow a reduction of Me_2SO concentration has not been demonstrated in hepatocytes although it has been successful with other cells [63]. This may be due to the presence, in the CPA medium, of other polymers such as albumin, serum or other impermeable molecules.

c) Cryopreservation/thawing protocols.

Crucial to the outcome of cryopreservation are the cooling and warming rates. Slow freezing protocols that allow avoidance of intracellular ice formation are most common for adult hepatocyte cryopreservation. For the same purpose, at the stage of re-warming most researchers use rapid thawing at 37-40°C.

Seckiguchi et al [107] have shown that during the freezing of isolated hepatocytes down to -196°C at a rate of 1°C/min with 1.5 M Me_2SO and rapid thawing, the viability of cells after a month of storage in liquid nitrogen was 60% of the control value. Similar results were obtained in another study using slow freezing and rapid thawing of suspensions in the presence of 1.5 M Me_2SO [100]. Very similar viability (50-65%) was obtained [23] after cryopreservation of human hepatocytes in medium containing 10% Me_2SO and 20% fetal calf serum using a programmed freezing rate of -1.9°C/min from 4 to -30°C and then -30°C / min from -30°C to -150°C.

Various metabolic processes of liver parenchymal cells have varying degree of cryosensitivity. Slow freezing of isolated liver cells (2°C/min) described in [94] yielded a high percentage of surviving cells frozen to -196°C; however, on closer inspection, the same freezing protocol applied to -30°C resulted in a number of cells with multiple morphological defects [35]. The rate of urea synthesis was depressed by 50% of freshly isolated cells. Freezing at a rate of 50°C/min causes significant reduction of viability of the suspension. Consequently, slow freezing rates are considered more appropriate for hepatocyte preservation.

The problem with slow cooling rates is the extension of time that cells spend at relatively high subzero temperatures in the residual liquid fraction prior to achieving the ‘quasi-

vitreous' state at ultra-low temperatures. During this time cells are exposed to high salt concentrations, and toxic CPAs. Besides, slow cooling allows sedimentation of cells on the bottom of the vials, where cell density become higher than the optimal, resulting in additional cell damage. According to metabolic parameters like cytochrome CYP3A4 activity, lactate dehydrogenase release and bilirubin conjugation measured after rewarming, cell concentrations from 10^6 to 10^7 cells/ml proved to be optimal [125]. However, De Loecker et al [17, 18] suggested that even lesser cell density of hepatocytes may increase hepatocyte viability after thawing. These results indicate that unless a high cell density of cells are specifically needed; samples with a low cell count (less than 10^7 cells/mL) are preferable.

Several methods are available for preventing sedimentation of cells: mixing samples at the initial stage of cooling [14], seeding [33] and using a rapid two-step cooling protocol.

The importance of ice seeding has been shown in study [100]. Isolated hepatocytes were equilibrated for 5 min with 0.5 M Me_2SO at room temperature, placed in a bath at 1-2°C below the ice nucleation temperature and induced ice seeding. Samples were cooled at various rates down to -60 ° C and then transferred to liquid nitrogen. The most effective were the cooling rates in the range from 0.5°C/min to 10°C/min. The synthesis of urea after rewarming was maintained at 50% and protein synthesis rate was only 25% of the freshly isolated cells.

A rapid two-step cooling protocol first developed for fibroblasts [30] was adapted for rat hepatocyte cryopreservation with 10% Me_2SO [81, 98]. For successful application of the protocol it was important to discover a safe temperature range which, on one hand, permitted rapid and complete crystallization of extracellular medium but, on the other hand, prevented supercooling of liquid, which promotes formation of intracellular ice crystals, lethal for cells. The best temperature range was found to be between -20°C and -25°C. This protocol has been shown to significantly maintain viability, gluconeogenesis, mitochondrial and ion-transporting functions and (CYP)-dependent xenobiotic biotransformation in a wide range (5×10^6 - 5×10^7 cells per ml) of hepatocyte densities.

4. CURRENT LIVER CELL BIOPRESERVATION: CRYOPRESERVATION IN ALTERED FORMATS - ENCAPSULATED OR SANDWICH CONFIGURATIONS.

One important consideration in translational studies using isolated hepatocytes for drug or metabolic investigations depends upon the nature of the in vitro culture systems which are applied; it has been known for some time that expression of normal liver phenotype may be best supported if the hepatocytes are provided with matrices which can maximise cell-cell contact in either 3-dimensional configurations or sandwich culture [90]. These ideas have been translated through to cryopreservation protocols, which then often require the low temperature preservation steps to be undertaken in receptacles such as multi-well tissue culture plates.

Sandwich culture where liver cells can be cultured between two layers of matrix such as collagen was developed more than 20 years ago, and early attempts to cryopreserve hepatocytes in this format produced low survivals [8]. One of the earliest successful reports of this approach was made by Koebe and colleagues [50] cryopreserving rat hepatocytes which had previously been delivered into a sandwich culture between two layers of collagen. The chosen CPA was 15% Me_2SO, and the tissue culture flasks were cooled at a slow rate to -70°C, with subsequent storage at -100°C. Rewarming was rapid, and some recovery of albumin secretion was noted, although lower than for control cells. The study was extended by Borel-Rinkes et al [9], who used a similar culture configuration and cooling rates of -5°C/min and an end temperature of -80°C, when 70% of pre-cryopreservation levels of albumin secretion could be obtained. Koebe went on to develop a specifically-designed

cooling chamber where porcine hepatocytes in sandwich culture could be cryopreserved in tissue culture flasks [49]. Incubation with CPA (10% Me_2SO) was performed for 15 min at 37°C, before cooling at either -1°C or -10°C/min to -80°C as an end temperature; the flasks were held in storage for either 3 or 30 days, before rapid rewarming and CPA dilution in stepwise manner at 4°C. Both albumin secretion and drug metabolism were measured, and it was shown that slow cooling (-1°C/min) was superior to faster (-10°C/min) rates, whilst the position of the flasks within the cooling chamber was important (attributed to different impacts of the cold nitrogen vapor pumped into the chamber). With the optimal cryopreservation protocol, storage for up to 30 days did not affect outcomes. The same group [51] went on to refine the technology for the cooling chamber, and reported 75% recovery of functions in porcine hepatocytes stored for 14 days at -80 °C.

Cryopreservation in the sandwich configuration has recently been applied to porcine hepatocytes in a flat membrane bioreactor configuration which might be suitable for a liver support device [39]. Slow cooling (-1 °C/min) with 10% Me_2SO was again selected with an end temperature of -80°C. Storage for a maximum of 14 days was carried out, with rapid rewarming and step-wise CPA dilution. Albumin secretion, urea synthesis and ethoxy-coumarin activities were recovered to about 60% of non-frozen controls by days 11 or 14 of storage.

More recently it was shown that, whilst monolayer (single layer gel) cultures of hepatocytes could be cryopreserved under similar conditions, in this format, the cells lost up to 50% of reduced glutathione content when recovered after 24h from -80°C [120]. Inclusion of antioxidants during cryopreservation reversed this trend, but not completely.

Cryopreservation of rat hepatocyte monolayers was also reported for cells cultured on collagen vitrigel membranes, produced by dehydrating collagen gels to a glassy state on a supporting matrix [84]. Slow cooling at -1°C/min after exposure to 10% Me_2SO was followed by storage in liquid N_2 and rapid rewarming. Much higher recoveries of attached viable cells were reported for vitrigel compared to cryopreservation of monolayers on normal plastic culture plates, and on vitrigel, the liver cells maintained typical polygonal morphology.

Another approach to cryopreservation of liver cells in 3-D configuration has been to either attach the hepatocytes onto microcarrier beads, or encapsulate the cells in alginate microspheres, relying on the adherence of functional cells, or the ability to mix cells and alginate before expulsion as droplets into a polymerizing buffer. Early studies focused on cryopreservation of adult hepatocytes attached to alginate-poly-L-Lysine-collagen microcarriers for liver cell transplant studies [21], with evidence of metabolic activity of the cryopreserved transplants by correction of hyper-bilirubinemia. Mahler and colleagues [70] encapsulated rat hepatocytes in 2% alginate micro-beads. Cryopreservation was carried out by a 2-step cooling method (with holds at -20°C and -80°C before transfer to liquid nitrogen), Me_2SO at 16% and rapid warming. Results showed that viable cell recoveries were about 85% of fresh cell numbers, whilst ethoxy resorufin de-ethylase and glutathione transferase activities were well preserved. Stimulation of post-thaw apoptosis was greatly reduced by alginate encapsulation compared with that seen in hepatocytes cryopreserved in free cell suspension. Aoki et al [4] reported alginate encapsulation of rat or human hepatocytes, in 1.5% alginate, and finally coated with poly-L-Lysine. Cryopreservation was carried out using 10% Me_2SO in 1.8 ml cryovials, rapid immersion in liquid nitrogen, and rapid rewarming. Morphology of the encapsulated hepatocytes was well maintained, urea synthesis was about 90% of control values after cryopreservation, and expression of CYP 450 enzymes at a transcriptional level was demonstrated for the microspheres cultured for up to 7 days after thawing. When the thawed encapsulated cells were used in a hepatocyte transplant model, function could be demonstrated throughout 1 week of studies. The same group [126] went on to show that uptakes of drugs such as salicylate, allopurinol and prostaglandin were well

preserved in thawed encapsulated cells, which had been cryopreserved for up to 120 days and held in liquid nitrogen.

Hepatocytes can also be induced to form 3-D aggregates by rotary culture, in the absence of added matrix. Lee and colleagues [60] produced rat hepatocyte spheroids, and exposed them to 10% Me_2SO as CPA in 4 different base media (UW solution, Williams E medium, fetal bovine serum or a mixture of all these). A programmed slow cooling profile was applied. Although data on ultrastructural stability of the thawed aggregates were not reported, UW solution proved to be the best CPA carrier solution when assessed by the MTT viability assay and albumin secretion.

In some cases, alginate encapsulation has been performed after cryopreservation as a way to either stabilize the thawed cells, or provide a matrix for cell transplantation. Son et al [117] cryopreserved rat hepatocytes in cell suspension using a 2-step cooling regime (-20°C, then -80°C before transfer to liquid nitrogen), and after rapid rewarming, immobilized the cells in 2% alginate supplemented with collagen and chitosan. The encapsulated thawed cells showed higher levels of urea synthesis than hepatocytes thawed and cultured directly.

Alginate encapsulation and cryopreservation has also been promoted for liver cell lines such as HepG2 cells, where large numbers of cells may be needed for bioartifical liver support. Cryopreservation of alginate-encapsulated liver cell spheroids produced by culturing HepG2 cells within 1% alginate was carried out using a slow cooling regime (-2°C/min), 10% Me_2SO as CPA, and storage in liquid N_2 [77]. Good recoveries of viable cell numbers, protein secretion and broad spectrum CyP 450 functions were reported. The work demonstrated the importance of control of ice nucleation ('seeding') during the cooling phase which reduced cryopreservation-induced apoptosis and improved viabilities which were tracked in culture up to 72h post-thawing. Albumin synthesis was also recovered after cryopreservation of the alginate-encapsulated liver cell spheroids after cooling using an electrically-powered cryo-cooling machine based on Stirling motor technologies [78].

5. BIOPRESERVATION OF LIVER CELL LINES AND HEPATIC PROGENITOR CELLS.

A number of liver cell lines, mainly of hepatoblastoma derivation, are available (including HepG2, C3A, and HuH-7 lines). As continuously-growing transformed cells, they present much less of a problem for cryo-banking, and they are routinely cryopreserved in many laboratories using simple slow cooling methods and Me_2SO as CPA. Regulated sources of HepG2 cells are routinely cryo-banked and supplied by agencies such as European Collection of Cell Cultures (ECACC), and little specific focus has been paid in recent times on details of the cryopreservation protocols. Some information does exist where the cell lines have been used for a specific purpose.

Shoji [110] and colleagues developed a cytotoxicity assay method which included cryo-banking HepG2 cells attached to collagen substrate in microtiter plates. The CPA was 10% Me_2SO, with slow cooling (about -1°C/min) to an end temperature of -85°C. End storage temperatures of -4 or -20°C provided functional cells after 2-4 days of storage, but only -85°C was adequate for long-term (30 day) storage. Zhu [136] and colleagues applied cryopreservation to HepG2 cells which had been transiently transfected with a specific drug receptor, as a way to cryo-bank samples and control large volumes of the cell product. The authors found that the cryopreserved transfected cells functioned as well as fresh transfected cells, when applying a slow cooling protocol with 10% Me_2SO and 20% fetal bovine serum in the CPA solution, an end temperature of -80°C and rapid warming. Menze and colleagues [82] investigated a pre-conditioning strategy before cryopreservation of a number of cell lines, including the liver subclone HepG2/C3A cells, applying slow cooling (-1°C/min) and ice

‘seeding’ at -6°C, and found that the metabolic preconditioning allowed a higher recovery of C3A cells.

In experimental models, which induce liver damage and simultaneously block hepatocyte proliferation, the recruitment of a hepatic progenitor cell population comprised of oval cells is invariably observed. Hepatic oval cells involved in some forms of liver regeneration express many markers also found on hematopoietic stem cells (HSCs). There is a substantial body of evidence to suggest that oval cells are involved in liver regeneration, as they differentiate into hepatocytes and biliary cells. The supply of human hepatic stem cells (hHpSCs) and other hepatic progenitors has been constrained by the limited availability of liver tissues from surgical resections, rejected organs from organ donation programs, and by the need to use cells immediately.

To facilitate accessibility to these precious tissue resources, recently there has been published an effective method for serum-free cryopreservation of the cells, allowing them to be stockpiled and stored for use as an off-the shelf product for experimental or clinical programs. The method involves use of buffers, some serum-free, designed for cryopreservation and further supplemented with hyaluronans (HA) that preserve adhesion mechanisms facilitating post thaw culturing of the cells and preservation of functions [127]. However, the bulk of published data on the role of bone marrow stem cells in liver damage suggest that they do not play a significant physiological role in replacement of epithelial cells in any known form of hepatic injury. Fully functional bone marrow-derived hepatocytes indeed exist, but are extremely rare and are generated by cell fusion, not stem cell differentiation [22].

There is currently an increasing interest in applications of liver progenitor cells across a range of scientific areas including both regenerative medicine and biotechnology, which raise a need for cryo-banking. Historically, fetal liver cells have been seen as important progenitor cells. The features of fetal tissues compared with adult tissues are a high proportion of stem cells, as well as a relatively small proportion of completely differentiated cells.

Fetal liver is formed from the derivatives of different germ layers and contain hematopoietic, hepatic, endothelial and stromal cells of different degrees of maturity [95]. The proportion of the hepatic cells increases whereas percentage of hematopoietic cells decreases during prenatal development. The complex cellular hierarchy of fetal liver raises the potential issue of different responses of its’ constituent cells to the cryopreservation procedure.

Within the cellular hierarchy of fetal liver the largest clinical potential have hematopoietic stem / progenitor cells, which have high self-renewal capacity and low immunogenicity [62, 86]. Furthermore, human fetal liver also contains committed hematopoietic progenitors, mainly erythroid precursors of different maturation stages, which could be valuable in the treatment of different forms of anemia. Therefore, the majority of protocols used for the cryopreservation of fetal liver cells is aimed at maintaining the pool of hematopoietic cells and are based on protocols developed early on for the hematopoietic bone marrow derived cells.

Usually, human fetal liver hematopoietic cells are cryopreserved by using slow cooling rates under the protection of 5–10% Me_2SO, supplemented with fetal serum [123]. This protocol allows one to save the majority of hematopoietic progenitor cells. Modification of the cryoprotective medium by introducing a non-penetrative sugar such as sucrose, enables a reduction in the concentration of Me_2SO, a reduction or replacement of serum and an increase in the efficiency of cryopreservation [99].

Suggestions for improvement in the protocol by adding membrane stabilizers and antioxidants have been made in a research study [61] where both the hematopoietic fraction from fetal liver and cord blood cells (another potential progenitor cell source) were studied. The authors used end-storage temperatures of -80°C and -196°C, and reported some

differences in efficacy of different additives. However, this cryopreservation protocol was ineffective to preserve fetal liver stromal cells, which are an important source of multipotent mesenchymal stromal cells (MSCs). It has been shown [112], that stromal cells in primary fetal liver suspension are dramatically sensitive to the cryopreservation procedure, in particular to supercooling. Only the addition of ice-seeding to the 3-step freezing protocol allowed it to protect colony-forming activity and the capacity for multilineage differentiation of MSCs.

Data on the response to the cryopreservation procedure of hepatic cells in fetal liver is absent from the literature. Their sensitivity can be judged only indirectly. Thus, it was shown [99] that the cryopreservation of human fetal liver cells using 5% Me_2SO resulted in a 15% decrease in viability of $CD34^+$ cells, as assessed by 7-AAD staining. Very similar reduction in viability was obtained after cryopreservation under the same conditions of glycophorin A-positive cells containing as its main population (about 90%) hematopoietic cells in fetal liver [124]. However, alamar blue reduction rate indicated that the viability of all cells in fetal liver was decreased 2 fold. Taking into account that the percentage of hepatic cells in human fetal liver in the 1^{st} gestation trimester of gestation is second only to that of hematopoietic cells and far higher than for other cell types, we can assume that they, at least, are more sensitive to cryopreservation procedure than hematopoietic cells. Of course, to answer this question a special investigation is necessary.

6. ALTERNATIVE APPROACHES TO LIVER CELL BIOPRESERVATION: VITRIFICATION TO AVOID ICE FORMATION; SUBZERO NON-FREEZING PRESERVATION; PRESERVATION BY DRYING.

Whilst cryopreservation has been the mainstay of true long-term storage of hepatocytes over the past decades, some alternative approaches have been researched to improve outcomes by avoiding the presence of ice altogether. These can be grouped into: a) vitrification; b) sub-zero non-freezing storage, and c) preservation in the dry state.

a) Vitrification

The process of vitrification involves cooling to deep cryogenic temperatures after exposing the hepatocytes to high concentrations of CPA (in the region of 40-60% w/v) with subsequent rapid cooling to avoid ice nucleation. Typically, fast warming rates are also necessary to avoid ice nucleation during the rewarming phase. So far, few groups have investigated vitrification for hepatocytes [119]. LL Kuleshova, et al [55] have used microencapsulated hepatocytes as a model to develop a method of vitreous cryopreservation for large quantities of cell-containing constructs. This procedure involves the idea that the scaffold and matrix are expected to support cell colonization, migration, growth and differentiation, and to guide the development of the required tissue, liver in this case. Cryopreservation can be achieved by conventional freezing and vitrification (ice-free cryopreservation). By using sucrose, a sugar that does not penetrate the cells, in combination with ethylene glycol, they were able to cryopreserve up to $5x10^6$ hepatocytes. The method included a pre-equilibration procedure in which the amount of penetrating cryoprotectant was gradually increased by 15% in each step [133]. The optimal vitrification solution consisted of 40% ethylene glycol and 0.6M sucrose. Three cooling rates (400°C/min and above) and three warming rates (650°C/min and above), in combination with the proposed vitrification solution, were equally effective. Optimization of the procedure and solution allowed microencapsulated hepatocytes to be preserved with almost 100% retention of cell functions and no detectable damage to the fragile microcapsules [55].

By applying this vitrification process, these authors were able to cryopreserve hepatocytes in tissue engineering constructs, showing a good response to membrane integrity and avoiding the usual apoptotic pathway triggered by cell detachment known as “anoikis”, a key contributor to the rapid decrease in viability of primary mammalian hepatocytes after isolation [67].

b) Subzero Non-freezing storage.

To date, few studies of hepatocyte preservation methods utilizing subzero nonfreezing temperatures have been reported [40, 79, 104].This research is based on previous investigations on nonfreezing storage of kidneys [12], heart [3, 106, 135] and liver [108, 116]. The general approach is the storage of isolated hepatocytes at temperatures between 0°C and the freezing point of the sample ($-T_{sample}$ °C). This method was originally reported by Matsuda et al [80] using UW solution at -4°C. They preserved rat hepatocytes up to 48h in a computer-programmed incubator which prevented ice formation in the -4°C range. They have found improvements in the TB exclusion test, ATP contents, MTT assay and urea synthesis during the rewarming of subzero nonfreezing group with respect to the UW (0°C) group. The lactic acid production was suppressed in the subzero nonfreezing group with respect to the 0°C preserved group. The authors claimed that the subzero temperature protects hepatocytes against hypoxic injury by suppression of cellular degradative catabolism in comparison with conventional hypothermic preservation [80].

There are some limitations in the application of this method, because at subzero temperatures without cryoprotective agents (CPA) the sample is in the supercooled state in which the preservation solution is below the equilibrium freezing point but not yet frozen. This metastable state poses the hazard of initiation of ice nucleation and freezing of the sample at any time due to a slight impact or change in temperature. The addition of a CPA to the preservation solution prevents ice formation and decreases the freezing point of the solution. In this temperature range [$\Delta = 0°|C - T_{sample+\ CPA}$ °C] the solution is not susceptible to ice nucleation, providing a safety zone for subzero nonfreezing temperature for cell/ organ preservation. In this way, Rodriguez and Guibert [40, 104] have developed a methodology to preserve rat hepatocytes at -4°C using 1,4-butanediol as CPA and UW solution. The first approach was to develop a Gas Chromatography method to estimate the concentration of 1,4-butanediol in the cells [2]. After that, it was determined: a- the effects of acute CPA exposure at 37°C to explore the possibility of cryoprotector toxicity, and b- the penetration of the CPA into the hepatocytes at -4°C. The CPA was added to the hepatic cells suspended in UW solution at a low rate (0.16 g/min /110 $.10^6$ cells in 36 mL of UW) to get a final concentration of 8% 1,4-butanediol. Then the hepatocytes were stored at -4°C up to 120 hours. The cellular concentrations of glutathione, ATP content, glycogen and lactate production were determined and compared with the results of conventional storage at 4°C. The results showed a significant increase of ATP content, a considerable glycogen and glutathione retention and suppression of lactic acid production in the cells stored at -4°C. These cells subjected to 60 min of rewarming in Krebs-Henseleit resuspension media, maintained a viability similar to fresh hepatocytes tested by propidium iodide test [38], glutathione and ATP content and functional tests as MTT assay and ammonium removal efficiency (ARE). The activity and gene expression of the urea cycle enzymes Carbamyl Phosphate synthetase I (CPSI) and Ornithine Transcarbamylase (OTC) were also tested. Although the cells preserved at -4°C showed initially, in the rewarming, diminished CPSI and OTC relative gene expression levels, they were able, after 60 min, to return to values comparable to the controls. Also, after 60 min of rewarming the ARE of preserved (-4°C) hepatocytes did not show differences with control group.

In the end, these cells were appropriate for In vitro studies or bioartificial liver devices; but not for hepatocellular transplantation, where an accumulation of macrophages were seen around transplanted cells in recipients livers (personal observations).

c) Storage in the Dry State

In Nature, some organisms have developed strategies to enable them to survive for long periods at ambient temperatures in a desiccated state. This involves a complex phenotypic response to environmental changes involving intracellular accumulation of cytoprotective agents such as the disaccharide trehalose and amino acids [16]. However, transferring this approach to mammalian cells raises several biological problems, which include how to introduce such sugars into the intracellular compartment. Few studies have, so far, been made in hepatocytes using such an approach, but Toner's group [1] investigated a switchable cell membrane pore to allow diffusion of high concentrations of trehalose into rat hepatocytes. This allowed accumulation of trehalose up to 0.5M, but work on long-term dry storage has not yet been reported.

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