SZENT ISTVÁN EGYETEM
ÁLLATORVOS-TUDOMÁNYI DOKTORI ISKOLA

Cryopreservation of late preimplantation stage mouse embryos, with a special emphasis on introducing hydrostatic pressure into the freezing protocol

Dr. Pribenszky Csaba

Doktori értekezés

Budapest
2004

Szent István Egyetem
Állatorvos-tudományi Doktori Iskola

Témavezető és témabizottsági tagok:

.....
Prof. Dr. Cseh Sándor

Prof. Dr. Solti László

Prof. Dr. Huszenicza Gyula

Készült 8 példányban. Ez a 1. sz. példány.

…….
Dr. Pribenszky Csaba

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AFP	antifreeze protein
CP	cryoprotective agent
CSP	cold-shock protein
DMSO	dimethyl sulfoxide
EG	ethylene glycol
FCS	fetal calf serum
GLY	glycerol
hCG	human serum gonadotropin
HHP	high hydrostatic pressure
HSP	heat-shock protein
IVF	in-vitro fertilization
LN_2	liquid nitrogen
OPS	open pulled straw
PEG	polyethylene glycol
PG	propylene glycol
PMSG	pregnant mare's serum gonadotropin
PVP	polyvinylpyrrolidone
RT	room temperature
SA/V	(surface) area/volume ratio
SSV	solid surface vitrification
ZP	zona pellucida

I. INTRODUCTION

„In February 2001, foot-and-mouth disease outbreaks occurred in the United Kingdom. Just a few weeks later, the infection had spread throughout England and cases were reported in the Netherlands, France, and the Republic of Ireland. Attempts to control and eradicate the epidemic resulted in the slaughter of over 4 million head of livestock in Great Britain alone. The outbreak of the disease in other parts of the world (i.e., Taiwan in the mid 1990s) and attempts to control and eradicate the disease have caused the unintentional loss of germplasm resources worldwide." – summarized Dobrinsky in 2002 the recent headlines from the world press.

The disastrous events justify the importance of assisted reproductive technologies including embryo cryopreservation. If embryos had been cryopreserved and held in long-term storage, permanent germplasm losses could have been prevented and lines could have been regenerated through embryo transfer after the epidemic.

The advancements in cryobiology, cell biology, and domestic animal embryology have enabled the development of embryo preservation methodologies for the domestic animal species, including cow, sheep, goat and recently swine, as well as for other species including the human.

Embryo cryopreservation provides permanent preservation technique for germplasm, breeding line regeneration or proliferation, global genetic transport, increased selection pressure of herd genetics, and methodology for genetic resource rescue; as Solti et al. (2001) also pointed out its economical and ecological importance in the preservation of indigenous livestock.

The aim of the presented experiments were to improve the efficacy of the cryopreservation of embryos being in late preimplantation stages such as expanded, hatching or hatched blastocysts.

For the trials, we used mouse embryos. Originally, our target species was the cattle, but due to the severe reduction of the Hungarian livestock from the late nineties (referring to the aforementioned thoughts), we encountered permanent difficulties in the cattle embryo supply; consequently we switched to employ mouse embryos instead. The conclusions obtained with mouse embryos are of importance in the future experiments to be conducted with other species as well.

Our experimentations are divided into two separate fields of research:

I. Examination of in vitro survival and in vivo developmental capacity of zona pellucida free, hatched blastocysts cryopreserved by rapid freezing with different cryoprotectants using different freezing and thawing protocols.

II. Study the possible beneficial effects of high hydrostatic pressure (HHP) in the process of embryo-(cryo)preservation based on the following two separate hypothesises:

    1. HHP reduces substantially the freezing point of the pressure medium. Based on this phenomenon, we tried to build up a protocol, through which we would be able to keep and preserve embryos in their optimal culture medium, under pressure, unfrozen even around – 21 °C.

    2. HHP induces the production of shock proteins in biological systems. Based on the alleged principle of cross-protection we tried different HHP treatments prior to the procedure of freezing of blastocysts in order to improve their ability to resist the “shock” of freezing.

In the following chapters, I summarize the history of embryo-cryopreservation, the physico-chemical and biological aspects of freezing, the different methods for cryopreserving embryos, the effects of high hydrostatic pressure on the biological systems and its practical uses in cryobiology followed by our own experiments.

II. LITERATURE REVIEW

1. A BRIEF HISTORY OF EMBRYO TRANSFER AND CRYOPRESERVATION

The science of reproductive biology paid tribute to the centenary of the first embryo transfer- related experiments a bit more then a decade ago, commemorating the pioneer of reproductive biology, Walter Heape, who reported the concept of embryo transfer in rabbits. More than half a century later, in 1953, Willet published a study about the successful transplantation of bovine embryos. Embryo transfer techniques have been developed in other species as well, though up until the early seventies embryo transfer was mainly a tool to investigate different processes in reproductive biology.

Christopher Polge used glycerol as the cryoprotectant in his successful sperm freezing experiments for the first time, in 1949. Cryopreservation of embryos first became a reality in the late 1960's. It was an idea that was spawned and brought to life by three researchers, Stanley Leibo, Peter Mazur, and David Whittingham. They were able to successfully preserve fertilized mouse eggs in liquid nitrogen at -196 °C, then thawed, and implanted them into surrogate mothers. Their pioneering technique was first published in *Science* of October 1972, the procedure involved precise timing, slow carefully controlled freezing and thawing for the survival of the embryos. The birth of the first frozen mouse pups was on June 17, 1972. In the same year similarly Wilmut reported successful results. Since then, hundreds of thousands mammalian eggs and embryos have been successfully cryopreserved and developed into normal live young capable of reproducing themselves.

Wilmut and Rowson achieved initial successful attempts in the cryopreservation of cow embryos, resulting in the birth of a calf, in 1973. The sheep was subsequently used experimentally as a model for the cow, and soon practical methods for both species were developed and later extended to the goat, horse and more recently to swine (Table 1.).

Table 1. The pioneers or those who performed the first successful cryopreservation of mammalian embryos.

Species	***Authors***
Mouse	Whittingham et al., 1972, Wilmut, 1972; Wood and Farrant, 1980; Kasai et al., 1981
Rat	Whittingham 1975
Rabbit	Bank and Maurer 1974
Cattle	Wilmut and Rowson 1973; van Wagtendonk, et al., 1994 (vitrification); Sommerfeld and Niemann, 1999 (ivp); Han et al., 2000 (transgenic); Keskintepe and Brackett, 2000 (icsi); Nguyen et al., 2000 (nuclear transfer)
Sheep	Willadsen et al. 1974, Willadsen 1977; Heyman et al., 1987
Pig	Nagashima et al., 1989, 1994, 1995; Dobrinsky and Johnson, 1994
Horse	Yamamoto et al., 1982, 1983; Skidmore, et al., 1991
Goat	Bilton and Moore 1976; Li et al., 1990
Cat	Dresser et al., 1988; Pope et al., 1994, 1997; Gomez et al., 2003
Primates	Pope et al., 1984; Balmaceda et al., 1986; Summers et al., 1987
Human	Trounson and Mohr, 1983; Lasalle et al., 1985

In addition, Leibo and Songsasen (2002) recently reviewed the cryopreservation of non-domestic species such as primates, non-domestic ungulates, buffalo, elephant, candies, felids and exotic species.

In Hungary, Haraszti and Ronay (1977) published results for the first time about oocytes transplantation in rabbit and sheep. The first calf originated from embryo transfer was born in 1978, by Hahn et al.. Solti and his colleagues conducted successful experiments concerning the cryopreservation of rabbit embryos in the early eighties, while Cseh was a pioneer in successful cryopreservation and transplantation of bovine and sheep embryos in 1983 and 1984 (Solti et al., 1981; Cseh et al., 1983, 1984). Vajta (1997) and Dinnyes (2000) improved the technique of vitrification by introducing the open pulled straw (OPS) and solid surface vitrification (SSV).

2. PHYSICAL AND BIOPHISICAL BACKGROUND OF THE FREEZING PROCESS

2.1. Freezing

Like many effects at equilibrium, freezing involves a compromise between minimising energy and maximising entropy. Molecules in ice have lower energy than they do in liquid water, because they more strongly attracted to their neighbours, but they also have lower entropy because they cannot move about. (Entropy is defined as the heat transferred in a reversible process divided by the temperature at which it is transferred. It has the dimensions of energy per temperature. In thermodynamics, the entropy therefore appears multiplied by temperature in such quantities as the chemical potential and the free energies.)

Solutes scarcely dissolve in ice, so the entropy of ice is unaffected by their presence. However, dissolved solutes in water increase the entropy of the water molecules, and so the compromise between lowering energy and maximising entropy occurs at a lower temperature (Franks, 1982).

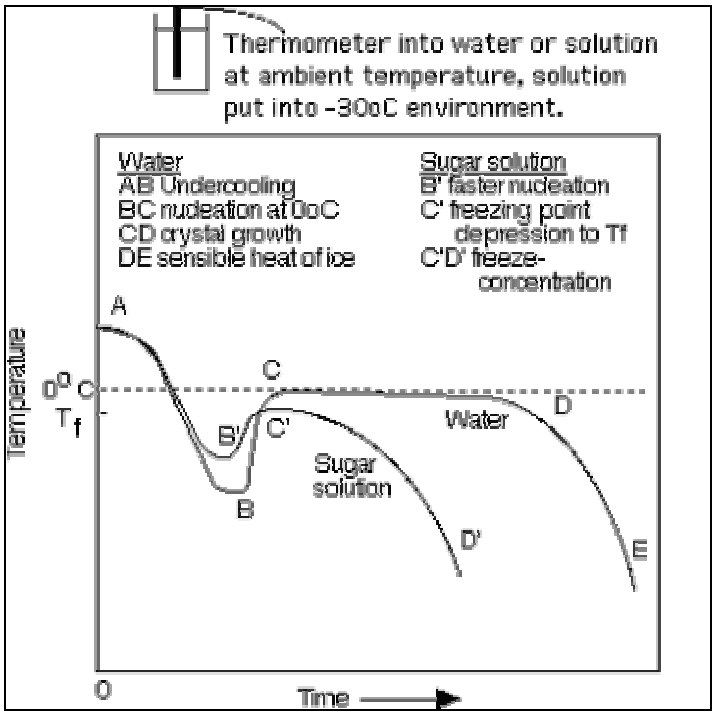


Figure 1. Time-temperature relationship for freezing of pure water and aqueous solutions.

Figure 1. shows the time-temperature relationship for freezing of pure water (ABCDE) and aqueous solutions (AB'C'D'). The first thermal event that can be seen from the diagram is supercooling below the freezing point before the induction of crystallization, from A to B or B'. This is a non-equilibrium, metastable state which is analogous to an activation energy necessary for the nucleation process. Pure water can be supercooled by several degrees before the nucleation phenomenon begins.

Once the critical mass of nuclei is reached, the system nucleates at point B or B' in the figure and releases its latent heat faster than heat is being removed from the system. In aqueous solutions, however, B' is not as low as B, since the added solute will promote heterogeneous nucleation, thereby accelerating the nucleation process. The temperature increases instantly to the initial freezing temperature, Tf, of the solution at Point C or C'. The presence of solutes results in depression of the freezing point based on Raoult's Law, which relates vapour pressure of the solution to that of pure solvent based on solute concentration. Note that C' is not as high as C, because the initial freezing point is depressed because of the solute. Hence, the solute has greatly decreased the amount of supercooling for two reasons: faster nucleation and lowered freezing point. In very concentrated solutions, it is sometimes even difficult to induce supercooling.

In pure water, the time line from C to D in the figure reflects the time during which crystal growth is occurring. Fast freezing rates promote the formation of many small ice crystals during this period. The partially frozen mixture will not cool until all of the "freezable" water has crystallized; hence, the line CD occurs at nearly constant temperature. The freezing time is usually defined as the time from the onset of nucleation to the end of the crystal growth phase. After crystallization is completed, the temperature drops from D to E as sensible heat is released.

During the freezing of the aqueous solution, a freeze-concentration process occurs as water freezes out of solution in the form of pure ice crystals (C'D'). This causes the freezing temperature of the remaining solution to drop. At temperatures well below the initial freezing point, some liquid water remains. In addition, a large increase in the viscosity of the unfrozen phase occurs, thus decreasing the diffusion properties of the system and hindering crystallization. It is more difficult to assign a freezing time to this process, but it is usually taken as the time to reach some predetermined temperature below the initial freezing point. The rate of freezing predicts ice crystal size - the faster

the rate - the more nucleation is promoted, and the greater number of crystals of smaller size that will result.

2.2. Nucleation and crystal growth (seeding)

All molecules in a liquid undergo Brownian motion. For freezing to occur in a – supercooled- liquid, the diffusing molecules must spontaneously form a small cluster of molecules (called nucleus, embryo or seed) which has a transient structure similar to that of the ice. In a supercooled liquid, such clusters form and dissolve rapidly. If however the cluster is larger than some critical size, it becomes energetically favourable for other diffusing molecules to join the structure, and it grows through the sample (crystallizing or freezing). Nucleation can be homogeneous (as described above) or heterogeneous, where an impurity (for example the wall of the straw) forms a substrate upon which nuclei can grow. All nucleations of supercooled biological solutions are heterogeneous. The term homogeneous should only be used for situations where great care is taken by employing, for example, ultra-pure water samples sequested in oil emulsions or levitation (Wilson et al., 2003).

The probability of nucleation occurring in a supercooled liquid depends on a number of factors: the probability increases

- with the volume of the sample,
- with the degree of supercooling and
- in the presence of impurities (which can act as heterogeneous nuclei).

The probability of nucleation decreases with increasing solution concentration.

A pure liquid in a small volume with no impurities can be supercooled a long way below its equilibrium freezing point. Small volumes (micro litres) of pure water, for example, can be cooled about – 40 °C (Wolfe and Bryant, 2001).

Nucleation can be induced externally. To avoid excessive supercooling and thereby to avoid ice nucleation of the embryos, crystallization can be induced in the extracellular medium 2 – 5 °C below the freezing point of the solution. Practically tweezing the straw containing the freezing medium and the embryos with a forceps previously dipped into liquid nitrogen adds a small “seed” crystal to the substance, which will cause it to solidify quickly. This induced ice formation in this slightly unstable, supercooled solution is called “seeding” (discussed also in the following chapters).

Without the initiation of extracellular ice formation the embryos do not dehydrate that will be detrimental during cooling.

2.3. Osmotic properties of the cell

Osmosis is defined as the flow of water across a semipermeable membrane from a lower solute concentration compartment to a higher solute concentration compartment. (Raymond, 1997).

A semipermeable membrane (cell membrane) is permeable to water but impermeable to solutes (macromolecules, proteins, sugar). Osmosis takes place because the presence of the solute decreases the chemical potential of water and water tends to flow from where the chemical potential is higher to where the chemical potential is lower. Decreasing the chemical potential of water lowers the freezing point of the solution as compared with pure water.

Osmotic pressure of a solution depends on the number of particles in solution, thus the degree of ionization of the solute must be taken into consideration.

Osmotic pressure is a colligative property of a solution, equal to the pressure that, when applied to the solution just stops osmosis. The cell can be, if somewhat simplified, an aqueous solution enclosed by a semipermeable membrane. Water will flow from the side of low solute concentration, to the side of high solute concentration. The flow may be stopped, or even reversed by applying external pressure on the side of higher concentration. This phenomenon is called reverse osmosis.

When water leaves the cell, the cellular solutes become more concentrated until the effective osmotic pressure of the cytoplasm is again equal to that of the extracellular fluid. In contrast to impermeable solutes, permeable solutes are those that are able to pass through the plasma membrane. Because of this ability, permeating solutes eventually equilibrate across the plasma membrane. Three rules help predict the volume changes cell will undergo when suspended in solutions of permeating and nonpermeating solutes:

1. The steady-state volume of the cell is determined only by the concentration of non-permeating solutes in the extracellular fluid.
2. Permeable solutes cause only transient changes in cell volume.
3. The greater the permeability of the membrane to the permeable solute, the more rapid is the time course of the transient changes.

The osmotic flow caused by a particular solute can be determined with the equation:
$Vw=L\Delta\pi$

- Vw rate of flow of water
- L a constant of proportionality=hydraulic pressure
- $\Delta\pi$ hydrostatic pressure difference.

This formula can be applied in case of non-permeating solutes.

Permeating solutes cause less osmotic flow. The greater the permeability of a solute, the less is the osmotic flow it causes.
The equation $Vw=\sigma L\Delta\pi$ takes solute permeability into account by including σ, the reflection coefficient, which is a property of a particular solute and a particular membrane, and represents the osmotic flow induced by the solute as fraction of the theoretical maximal osmotic flow (Raymond, 1997).

2.4. Solution behaviour during freezing and thawing

Freezing is a probabilistic process and, in most cases, since the extra-cellular solution has a larger volume than the intracellular solution, extracellular freezing occurs first. When it does, the extra-cellular solutes are concentrated in a small quantity of unfrozen water. This causes water to leave the cell: the characteristic time "t" (in tens of seconds) for hydraulic equilibration is: $d/(3L_pP_o)$

- d characteristic cellular dimension,
- L_p hydraulic permeability of the membrane and
- P_o osmotic pressure.

In this way, the cell dehydrates. Dehydration increases the osmotic pressure of the intracellular solution (the cytoplasm) which depresses its freezing point (Wolfe and Bryant 2001).

The phenomenon known as supercooling refers to the situation in which liquid can be temporarily cooled to below its freezing point without ice crystal formation. This freezing avoidance mechanism has the advantage that the solutions remain liquid and allow relatively normal, though slower, metabolism. It has the disadvantage that a supercooled solution is unstable with respect to coexisting phases of ice and a more concentrated solution (Scheider and Mazur, 1986).

Supercooling occurs when heat is removed from a liquid so rapidly that the molecules literally have no time to assume the ordered structure of a solid. At some lower temperature, ice nucleation occurs, followed by a rapid rise in temperature due to the release of latent heat of fusion. This process is lethal for the embryos not only because of the very rapid changes in the temperature, but also because the level of dehydration of the cells in the supercooled liquid is the same as at the freezing point.

At re-warming –thawing-, ice melts, the ice structure partially disintegrates, and the molecules become more compactly arranged, leading to a more dense liquid.

Because molecules are more strongly held in the solid phase, heat is required to bring about the solid-liquid phase transition until the melting point. At this point, the solid begins to melt and during the melting period, heat is absorbed by the system, yet its temperature remains constant. Once it has melted completely, the liquid temperature starts to rise again. There is influx of solution towards the intracellular compartment until equilibrium is reached again (Raymond, 1997).

2.5. Freezing rate and transport of water across the cell membrane.

The freezing rate is an important parameter in successful cryopreservation and post-thawing viability of the embryos, since improper rates allow injury to occur to the cells during cooling or thawing. Each type of cell has an optimal freezing rate, which provides the cell sufficient time to dehydrate. This process begins after the initiation of the phase transition of the extracellular liquid, when, due to the formation of ice, its osmotic pressure rises. The cell equilibrates by “transferring” water extracellularly through the cell membrane. The membrane, above -10°C, also plays an important role in preventing the passing of ice formation from the extracellular fluid to the intracellular area once the phase transition started. Thus together with the elevation of the extracellular osmotic pressure, supercooling occurs inside the cell (Mazur, 1980).

Equilibration of the cells to the increasing extracellular osmotic pressure can be achieved in two ways: 1) the intracellular water leaves the cell, or 2) the intracellular water freezes inside the cell (Mazur, 1980).

The ease of water transport depends on the membranes` permeability to water at any given temperature, the surface area-volume ratio of the cell and the freezing rate (Wolfe and Bryant 2001):

Larger cells (like embryos) have a relative smaller surface compared to smaller cells (like red blood cells); this is why their ability to dehydrate is reduced.
The flow of water through the cell membrane is greatly reduced in lower temperatures. Consequently, the time needed to transfer a standard unit of water through the cell membrane increases with decreasing temperature. The permeability of the different cells to water is affected by the temperature in a manner that is different from cell type to cell type. (On condition of the same small amount of reduction in the temperature, embryos increase the speed of water-transfer orders more, than red blood cells do.) The speed of water-transfer through the membrane raises with increasing difference between the intra- and extracellular concentration (Leibo, 1981, 1986).
The previously listed features define the optimal freezing speed, which can be different depending on the type of the cell. If the speed of freezing is faster then the optimal, the cell is not provided sufficient time to dehydrate, consequently the water will freeze inside the cell causing irreversible injuries depending on the amount and shape of the intracellular ice crystals (Mazur, 1980). If the speed of freezing is slower than the optimal, than severe dehydration and the so-called solution effect will be the causes for reduced survival (also mentioned in the next chapter).

3. CELL INJURIES DURING FREEZING

3.1. Intracellular ice formation and the solution effect

Sustained exposure to cryogenic temperatures (- 196°C) is not in itself dangerous: at this temperature, biochemistry and physiology occur at rates that are effectively zero. If cells are undamaged when they achieve the temperature of boiling nitrogen, little is likely to change if the samples are undisturbed and the temperature is maintained (Mazur, 1980; Leibo, 1981).
According to Mazur (1980), mainly intracellular ice formation and the solution effects cause damage to cells during freezing and thawing.

The injurious effects of intracellular ice are either caused by direct mechanical damage to the cellular ultrastructure or by the osmotic stress imposed on the cells. Osmotic stress occurs as the ice is converted into free water (melting), resulting in the transient exposure to a solution, which is hypertonic with respect to the inside of cells.

Embryos must absorb water from their environment to remain in osmotic equilibrium and to return to their normal isotonic volume. Because most metabolic reactions are arrested or drastically slowed during cryopreservation, they are especially susceptible to warming or dilution shock at this stage.

The solution effects mean the harm caused to the cells by the increased concentration of the intracellular solutes during dehydration. When an aqueous solution freezes, the ice is nearly pure, and the remaining unfrozen water is the solvent for all the solutes, at concentrations that become very large as temperature falls. These concentrations may be toxic. High concentrations of electrolytes affect ionic interactions, including those that help stabilize the native state of enzymes. Unfolding and denaturation of enzymes is often irreversible. Furthermore, ice and water interact differently with hydrophilic surfaces. This is potentially important because the surface tension of water is also involved in maintaining the native state of enzymes (Maurer, 1978; Wolfe and Bryant 2001).

The correlation between freezing rate and cell injury relies on two facts (Mazur, 1980):

- Damage from solution effects is minimized during fast freezing but at the same time, it leads to intracellular ice crystals formation. This is because water does not have enough time to diffuse out of the cell, and freeze internally creating crystals.
- Slow freezing leads to increased damage from solution effects because of the ample time for the water to osmotically diffuse out of the cell.

Based on these features, the optimum cooling rate should be rapid enough to minimize solution effects and at the same time slow enough to prevent intracellular ice crystals. Therefore, the optimal rate for the particular cells depends on their relative tolerance to damage from ice crystals and toxicity from solution effects.

During the process of slow cooling, two other possible injuries need to be mentioned:

Some cells rupture during freezing and osmotic contraction. Simple mechanical rupture by the advancing ice crystals is only one likely cause. Another cause of rupture may be electrical: the large transient electric field associated with the advancing ice front in a weak electrolyte solution can cause potential difference across the cells, which

is large enough to rupture membranes. The explanation lies in the different ability of dissolving ions of water and ice in the moving ice-water interface. As a result, the moving interface can generate a potential difference of tens or even hundreds(!) of volts, which is enough for the electrical rupture of the cell (Steponkus et al., 1985).

Cells can severely dehydrate in the high concentration suspending media (during freezing) in such a way, that when the solution is diluted (during thawing) they fail to expand osmotically. This cellular malfunction is associated with the loss of membrane semipermeability in the freezing-induced dehydrated state. Amongst the reasons, the changes in the lipid component of the membranes and the topological changes of the membranes are mentioned (Wolfe and Bryant, 2001; Gordon-Kamm and Steponkus, 1984).

3.2. Avoiding intracellular ice formation

3.2.1. Supercooling

Supercooling refers to cooling a liquid below its equilibrium freezing point, without freezing. This phenomenon was discussed earlier in detail. The importance of supercooling in cryopreservation is that it allows vitrification. Studies report the use of antifreeze proteins (AFPs) in cryopreservation of embryos, which non-colligatively lower the freezing point of aqueous solutions, block membrane ion channels and thereby confer a degree of protection during cooling (Baguisi et al., 1987). Antifreeze proteins were found for the first time in small molar concentrations in the blood of Antarctic fishes; they were first believed to only impend the growth of ice crystals (DeVries, 1984).

3.2.2 Intracellular vitrification

In cryopreservation, the usual goal is to achieve intracellular vitrification while avoiding intracellular ice formation and membrane damage. One of the deciding factors is the cooling rate. If a liquid is cooled sufficiently quickly, it can avoid freezing and vitrify (form an amorphous, glass phase). The necessary cooling rates are extremely high for pure liquids (e.g. 10 million degrees per second for pure water), but much more realistic for solutions. For aqueous solutions of typical cryoprotectants, cooling rates of about 10-1000 °C/min are sufficient to achieve vitrification.

At very low temperatures, the viscosity of a solution rises sharply, and molecular diffusion is reduced. If cooling is fast, then the viscosity rises rapidly, hindering nucleation. If cooling is sufficiently fast, the viscosity can become so large that molecular diffusion is effectively halted, and the probability of nuclei formation becomes negligible. The sample is then said to be a glass or vitreous solid, and the process is called vitrification. A glass is amorphous (unlike a crystal, it has no long-range order) but has the mechanical properties of a solid. A material is described as a glass if its viscosity reaches 10^{14} Pa s (Franks, 1982). A glass is by definition in a state of very long-lived non-equilibrium.

Vitrification provides an alternative approach for successful cryopreservation of embryos (discussed later, in the methods for cryopreservation section). When embryos are cryopreserved using conventional procedures, ideally intracellular vitrification happens even in the presence of extracellular ice. At the cryopreservation method first described by Rall and Fahy in 1985, embryos suspended in highly concentrated aqueous solutions of cryoprotective agents supercool to very low temperatures, obtaining both intra and extracellular vitrification.

3.2.3. Dehydration

Freezing (see earlier) usually occurs outside the cell first, because that is where there are more freezing nuclei, and because the extra-cellular solution has a larger volume than the intracellular solution. When this happens, the extra-cellular solutes are concentrated in a small quantity of unfrozen water, which necessarily has a higher osmotic pressure. This causes water to leave the cell. The characteristic time for water to flow out of cells under these conditions is tens of seconds (Wolfe and Bryant, 1992), so we refer to cooling as fast or slow according to whether substantial temperature changes are possible over this time scale. Cryopreservation usually uses fast cooling and so, when and if extracellular ice occurs, cells do not have time to dehydrate severely, although there is usually some dehydration of cryobiological importance. Dehydration increases the osmotic pressure of the intracellular solution (the cytoplasm) which depresses its freezing temperature and promotes vitrification: both inhibit intracellular ice formation.

At the conventional embryo freezing technique, the aim is to drive as much water as possible from the embryo and thus prevent intracellular ice formation while keeping the cytoplasm supercooled until freezing. After inducing phase change, the formation of ice brings an increase in the concentration of salts in the suspending solution. From this

time on the slow cooling process allows the embryos to respond osmotically to the concentration changes with further dehydration (Wolfe and Bryant, 2001).
During rapid freezing dehydration of the embryos also achieved before cooling by using a non-permeating cryoprotectants, such as sucrose, which creates a hyperosmotic environment.

3.2.4. Depression of the equilibrium freezing point

The accumulation of the solutes in the intracellular (and extracellular) solutions results in the depression of the equilibrium freezing temperature. Solutes, which can be tolerated in high concentrations (compatible solutes), include a number of sugars. High concentrations of such molecules increase the viscosity and thus reduce diffusion in solutions. This slows metabolism, but has advantages for slowing further dehydration. The main importance of freezing point depression in cryopreservation is in the avoidance of crystallization under conditions of low viscosity in relatively high sub-zero temperatures (Wolfe and Bryant, 2001).
A classical approach to freezing involved the gradual depression of the equilibrium freezing point during cooling to around -80 °C (Elford and Walter 1972; Fahy, 1980).
There is another method to reduce the freezing point of a solution, which is achieved by the application of high hydrostatic pressure: solutions can be maintained unfrozen even at low subzero temperatures by applying a certain pressure to them (Bridgeman, 1970). This phenomenon will be discussed later, in the hydrostatic pressure effects chapter.

4. CRYOPROTECTIVE AGENTS

Cryoprotective agents (CPs) are macromolecules added to the freezing medium in order to protect the embryos from the detrimental effects of intracellular ice crystal formation or from the solution effects, during the process of freezing and thawing.

There are two main groups of CPs according to their ability to diffuse through the cell membrane, namely the permeating CPs and the nonpermeating CPs (Hafez, 1993).

The permeating CPs added to the medium, lower the freezing point of the solution, increase the viscosity and thus reduce diffusion in solutions. The agents achieve the same effects permeating into the blastomeres, consequently retard dehydration of the cells: embryos have more time to dehydrate. In this way, embryos may be cooled slowly enough, to prevent the formation of large ice crystals intracellularly (Schneider and Mazur, 1984). Criteria for a cryoprotectant are high solubility, low toxicity at high concentrations and a low molecular weight both for easier permeation and for exertion of a maximum colligative effect, which is the reduction of the concentration of intracellular electrolytes (Hafez, 1993). Toxicity refers to osmotic stress and chemical toxicity on the cells. The cryoprotective agents reduce injuries in connection with the freezing, but also cause osmotic stress to the embryos (Leibo, 1981; Schneider and Mazur, 1984).

During the addition and dilution of a permeating cryoprotectant, the cell undergoes osmotic changes in size. Consequently, if the addition or dilution of the cryoprotectant is carried out inappropriately, the viability of cells can be affected. In general, when the embryo is exposed to a cryoprotectant it will initially shrink by losing water both because of the initial hyperosmoticity of the extracellular solution and because the embryo is much more permeable to water than to the cryoprotectant. Shrinkage will continue until the efflux of water is balanced by the influx of cryoprotectant. Concomitantly, water will re-enter the cell, forcing it to gradually increase its volume. Equilibration is complete when the embryo regains its isotonic volume (Wolfe and Bryant 2001). Previously, the cryoprotective agent was introduced gradually, step by step to the embryos, to avoid the detrimental effects originating from the sudden application of a substantial gradient of osmotic pressure (Bilton, 1980; Willadsen, 1980; Pettit, 1985; Wright, 1985). Experiments also have been published concerning the application of the cryoprotectant in one-step. According to Schneider and Mazur (1984) the one step dilution is well tolerated by bovine blastocysts.

The removal of the cryoprotectant following thawing is also not a negligible phase in the process of cryopreservation. When the external medium is abruptly diluted back to its original composition (the osmotic equivalent of thawing), the volume increases rapidly and hence the surface area increases almost equally rapidly. The plasma membrane can support a tension of only several mN m^{-1} without rupture, which is about 1 kPa hydrostatic pressure. The probability of membrane-rupture depends on the hydraulic conductivity and elastic modulus of the membrane, as well as on other features (Wolfe et al., 1986). Theoretically, the blastomeres of the embryos may expand excessively and rupture due to the rapid influx of water (water permeates into the cell much more rapidly, then the cryoprotectant permeates out of it), if the embryo –with maximal intracellular cryoprotectant concentration- is placed directly to the embryo culture medium.

According to the published reports, there are two main ways to remove the cryoprotectants from the embryos. Early reports suggest that the cryoprotectant may be removed in 0.25 M steps by transferring the embryos through PBS solutions of decreasing glycerol concentrations until they are in PBS with serum (Bilton and Moore, 1979; Pettit, 1985; Wright, 1985). In 1982, Leibo introduced a new method for removing glycerol by including a 0.25 M sucrose solution in the straw, postulating that glycerol would diffuse passively into the exterior of the cells, and sucrose, which could not permeate the cells, would prevent the osmotic shock during dilution. The high concentration (0.25- 1 M) of sucrose in the rehydration medium prevents the fast permeation of water into the cells. The cryoprotective agent gets extracellularly through passive diffusion, followed by the efflux of water: consequently, the cell shrinks. There are also methods that apply non-permeating cryoprotectants gradually at the process of the removal of the permeating cryoprotectant. Fahning and Garcia (1992) reviewed a number of trials utilizing any of the methods.

The permeating CPs most widely used are glycerol (GLY), dimethyl sulfoxide (DMSO), propylene glycol (PG), and ethylene glycol (EG). Each of these macromolecules has different biophysical characteristics, which determine their use and efficiency as cryoprotective agents. In addition, studies revealed that their chemical toxicity also varies (Hafez, 1993).

The first cryoprotectant used was glycerol, in 1949. Miyamoto and Ishibashi first applied ethylene glycol in 1977. The lower molecular weight of ethylene glycol compared to the others is what makes it highly permeable to the embryos and thus the

stepwise dilution with or without sucrose can be avoided (Bracke and Niemann 1995). Sommerfeld and Niemann (1999) who tested concentrations from 1.8 to 8.9M demonstrated its low toxicity.

The second group of cryoprotectants include the nonpermeating agents like sucrose, maltose, dextran, trehalose, sorbitol, albumin and acetamide. Their role is to stabilize and preserve proteins -the cell membrane- during the permeating cryoprotectant removal, improve the active transport of ions inside the blastocoel (Borland, 1976) and they have an excellent ability to form stable glasses.

Sucrose solutions were first used to reduce osmotic shock during cryoprotectant removal from frozen-thawed red blood cells. Sucrose treatment has since become a popular alternative to the classical step-wise removal of cryoprotectants from mammalian embryos. As discussed previously in this chapter, the sucrose acts as an osmotic counter force to restrict water movement across the membranes since sucrose is impermeable to cells. As the cryoprotectant is leaving the embryo, it will shrink in response to the extracellular hypertonic dilution medium. It regains its normal volume when at the end of the process the sucrose is removed from the dilution medium, thereby returning the medium to isotonic conditions. The observed shrinkage and swelling of embryos during a sucrose dilution treatment are preliminary indications that they have survived freezing because they demonstrate that the cell membranes function normally. Swelling of the cells, which usually can be seen between the steps in stepwise dilution, does not occur to as great an extent in sucrose solution. Several authors have reported an improvement in embryo survival after thawing in a sucrose gradient compared to the stepwise method (Niemann et al., 1982). Nonpermeating CPs also reduce the rate of permeation of other cryoprotectants (Niemann et al., 1982).

Additives with large molecular weights, such as disaccharides like sucrose or trehalose, do not penetrate the cell membrane, but they can significantly reduce the amount of cryoprotectant required as well as the toxicity of EG by decreasing the concentration required to achieve a successful cryopreservation of oocytes and embryos. The incorporation of nonpermeating compounds into the vitrifying solution and the incubation of the cells in this solution before any vitrification help to withdraw more water from the cells and lessen the exposure time of the cells to the toxic effects of the cryoprotectants.

Eroglu et al. published a novel method in 2000 concerning the intracellular use of non-penetrating cryoprotectants. They reported greatly improved post thaw survival of mammalian cells achieved by the introduction of intracellular trehalose. To overcome the impermeability of cell membranes to sugars, the authors previously used a genetically engineered mutant of wild-type Staphylococcus aureus haemolysin, which forms two nm transmembrane pores upon introduction to lipid bilayers. This engineered pole can be opened and closed by the removal or addition of micromolar concentrations of Zn^{2+} ions. By loading trehalose into the cells, the authors achieved increased survival after the cryopreservation of fibroblasts and keratinocytes.

The application of macromolecules may also facilitate the process of cryopreservation. The addition of a polymer with a high molecular weight such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), or Ficoll is sufficient to vitrify extracellularly with the same cryoprotective concentration used intracellularly. It was demonstrated that in certain circumstances, a polymer could reduce the minimal concentration of the cryoprotectant that is required for vitrification by 7% on average and by as much as 24% in combination with an increased hydrostatic pressure (Fahy et al., 1984). Several studies evaluated the potential beneficial effects of adding macromolecular solutes to the vitrification solution to facilitate vitrification (O`Neill et al., 1997; Dumoulin et al., 1994; Shaw et al., 1997). These polymers can protect embryos against cryoinjury by easing the mechanical stresses that occur during cryopreservation (Dumoulin et al., 1994). They do this through modifying the vitrification properties of these solutions by significantly reducing the amount of cryoprotectant required to achieve vitrification itself (Shaw et al., 1997). They also influence the viscosity of the vitrification solution and reduce the toxicity of the cryoprotectant through lowered concentrations.

Furthermore, the polymers may be able to build a viscous matrix for encapsulation of the oocytes/embryos and prevent crystallization during cooling and warming (Kasai et al., 1990; Kuleshova et al., 2001). Indeed, O'Neill et al. (1997) observed that addition of PEG resulted in greatly improved viability of oocytes following cryopreservation and vastly reduced the variability seen with vitrification solution alone. Shaw et al. (1997) were able to show that PVP, Ficoll, and dextran interacted with an EG-based vitrification solution with extreme variation. They concluded that Ficoll and dextran had little or no effect on the glass transition of the solutions. In contrast, with the presence of

PVP, the melting temperature and the glass transition temperature of the vitrification solution increase with the PVP content of the solution (Liebermann et al., 2002).

5. METHODS FOR CRYOPRESERVING EMBRYOS

According to the published studies there are two basic approaches to cryopreservation of cells, both of which have been used successfully to preserve a wide variety of cell types, including spermatozoa, oocytes, and embryos:

- Equilibrium cryopreservation by which cells are suspended in a relatively low concentration of a cryoprotective additive (CP) (around 10% ~ 1.5 M solution) and cooled at low rates of -0.5° to 2 °C/min. This cryopreservation technique include 1) addition of cryoprotectant, 2) cooling of the embryo, induction of ice formation, freezing and storage in liquid nitrogen, 3) thawing of the embryo, 4) removal of the cryoprotectant. The method allows permeation of cryoprotectant at room temperature and the dehydration of the embryos during the cooling and freezing processes before being plunged into liquid nitrogen.

- Non-equilibrium cryopreservation by which cells are exposed very briefly (usually, but not always) to high concentrations of a mixture of CPs (around 40% ~ 6 to 8 M solution) and cooled at high rates of >500°C/min.

5.1. Slow or conventional or "standard method" of cryopreservation (equilibrium cryopreservation)

The slow freezing method is the first method applied in the embryo cryopreservation. Slow rates of freezing are utilized protecting in this way the cell from intracellular ice formation. The other potential cell injury causes, namely the solution effects, are handled with the addition of cryoprotectant(s) in the freezing medium.
During the initial phase, the embryos are exposed and equilibrated to the cryoprotectant. Embryos exposed to a permeating cryoprotectant shrink by losing water until equilibrium is reached (discussed previously). As the cryoprotectant permeates the embryo, it gradually re-expands because of the re-entry of water, to maintain the osmotic equilibrium (Mazur and Schneider, 1986; Schneider, 1986; Schneider and Mazur, 1984). The rate of reexpandation depends on the species of the embryo, the stage of embryonic development, the embryos` surface area/volume ratio (SA/V), the

characteristic features of the cryoprotectant and the temperature of the exposure (Leibo, 1989).

During cooling to the temperature of the seeding (speed ranges from direct transfer to 5-12 °C / min), the aim is to dehydrate the embryos as much as possible thus preventing intracellular ice formation while keeping the cytoplasm supercooled. After seeding (temperature ranges from -4 to -7 °C), embryos are cooled further (speed ranges from 0.3 to 0.5 °C / min), after a possible holding time at the seeding temperature, to the plunge temperature (the temperature, where the straws containing the embryos are placed into LN_2; ranges from around -30 - -40 °C to -60 - -89 °C), allowing to respond osmotically to the concentration changes. The optimal cooling rate depends on the SA/V of the embryo again, and on the water permeability/temperature coefficient of the embryo. During this process a cattle embryo can shrink to 50% (at -15°C) or to 40% (at -20°C) of its original volume (Lehn-Jensen et al., 1982).

Embryos cooled slowly to around – 60 °C dehydrate more than those do, which were plunged to LN_2 at warmer temperatures (-30 - -40 °C): the latter ones still contain vitrified residual water. To avoid recrystallization, those with warmer plunge temperature need to be thawed rapidly (about 300 °C/min), while the ones with low plunge temperature are thawed slowly (about 20 °C/min).

The final step after thawing is the removal of the cryoprotectants from the embryos, which can be achieved by transferring the embryos to embryo holding solutions of decreasing cryoprotectant concentrations to reduce osmotic shock.

Whittingham reported the first successful mammalian embryo freezing in 1972 and the first calf resulting from the transfer of a frozen bovine embryo was born the following year. These early studies indicated that slow freezing of cattle embryos at low subzero temperatures (-80^{o}C) required slow thawing.

Subsequently it became evident that slow freezing could be interrupted at relatively high subzero temperature –25 to –35^{o}C before plunging into liquid nitrogen. In 1977, Willadsen modified the original method of Whittingham et al. to freeze and thaw mouse embryos. Using 1.5 M dimethyl sulfoxide (DMSO) as a cryoprotectant, Willadsen froze sheep and cattle embryos at a low rate of 0.3°C/min but only to an intermediate subzero temperature of -36°C instead of to -80°C, before plunging the samples directly into LN_2. Critical to the success of this method was that the frozen samples had to be warmed rapidly at 360 °C/min, rather than slowly. Significantly, Willadsen was able to produce

lambs and calves by transfer of embryos frozen by his modified procedure (Leibo and Songsasen 2002).

Since 1977, Willadsen's procedure has been widely adopted as a "standard method" to cryopreserve embryos of many species.

In the literature, much attention has been paid to the specific low cooling rate used to cool the embryos after seeding and to the specific intermediate subzero temperature to which the samples were cooled before they were placed into LN_2 for storage. The results in Fig. 2 suggest that these details of cooling rate and intermediate temperature are rather unimportant. The figure shows survival of 8-cell mouse embryos and of bovine blastocysts. In both cases, survival based on in vitro development is shown as a function of cooling rate. The mouse embryos were cooled at controlled rates to -80°C before being cooled to -196°C, whereas the bovine embryos were cooled only to -35°C before being plunged into LN_2. Despite these differences, it is clear that the response of embryos of these two very different species is very similar. It must also be noted that the volume of mouse embryos is 2.21 x $l0^5$ pm^3 with a surface area/volume ratio (SA/V) of 0.08. Bovine embryos have a volume of 1.77 x $l0^6$ pm^3 and a SA/V of 0.04. The higher the SA/V, the more rapidly does a cell dehydrate when exposed to hypertonic solutions, as occurs during cryopreservation. In principle, then, mouse embryos ought to tolerate cooling at rates much higher than bovine embryos do. However, despite the fact that mouse embryos have a SA/V twice that of bovine embryos, it is clear that this fundamental characteristic has only a relatively minor effect on the response of embryos to cooling rate (Leibo and Songsasen 2002).

Given the wide range of species and the differences between mouse and bovine embryos in their physical dimensions and physiological characteristics, these data taken together suggest that it ought to be possible to cryopreserve embryos of many species using this technique. This is not to say that this method will work with all species or that it will necessarily yield maximum survival. If the embryos are especially sensitive to chilling injury, as is true of porcine embryos or very early cleavage-stage bovine embryos (Leibo et al., 1996), then almost certainly slow cooling will produce poor or no survival. This slow-cooling approach will also result poor results with oocytes because of their extreme sensitivity to chilling injury (Zenzes, 2000; Parks, 1997; Parks and Rufting, 1992; Leibo et al.,1996; Bernard and Fuller 1996). However, embryos of many species are not damaged when cooled to near 0°C (Leibo and Songsasen 2002).

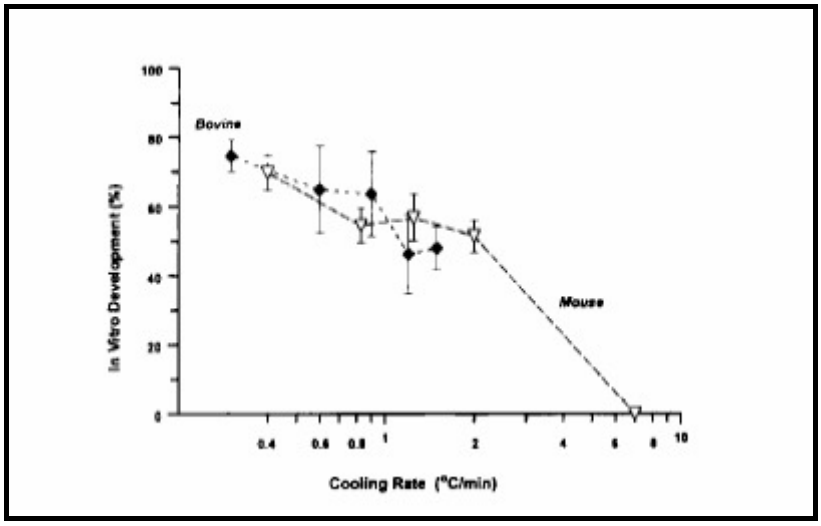


Figure 2. Survival of mouse embryos and bovine embryos as a function of cooling rate. The 8-cell mouse embryos were cooled at various rates to -80°C before being plunged into liquid nitrogen (LN_2); the data are those of Whittingham et al. (1972).The bovine blastocysts were cooled at various rates to -35°C before being plunged into LN_2; the data are those of Hochi et al. (1996) (Leibo and Songsasen 2002).

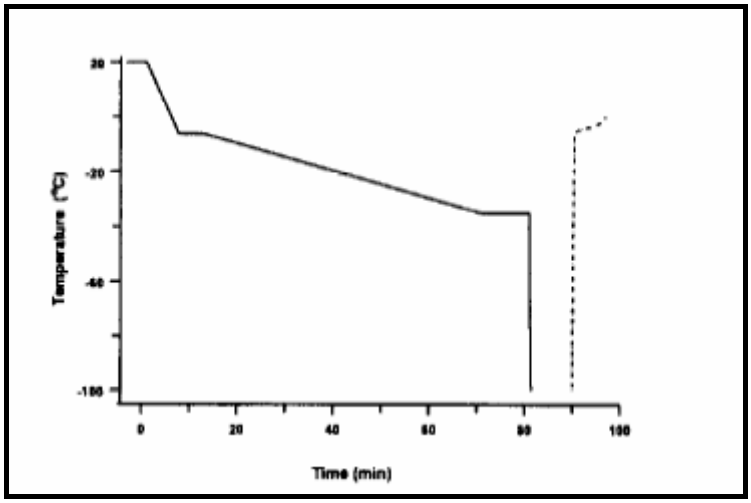


Figure 3. Relationship between temperature and time of the „standard method" for cryopreservation of embryos (Leibo and Songsasen 2002).

The embryonic stage that was considered to be the most suitable for freezing was the blastocyst stage.

Massip et al., in 1979 introduced the 0.25 ml plastic insemination straw as a freezing container. It allowed easy storage and offered the possibility to dilute the cryoprotectant within the straw by filling it with diluent (sucrose) and then cryoprotectant containing

embryos, which were separated from the diluent by air bubbles. After thawing the two fractions were mixed by shaking the straw before transfer (Leibo 1984.).

A simpler procedure avoiding the need for the dilution step after thawing was to incorporate sucrose in the freezing medium (1.36 M glycerol) at a concentration of 0.25 M (Massip and Van der Zwalmen 1984). The embryos exposed to the glycerol-sucrose mixture were pre-dehydrated prior to cooling; this means that slow cooling could be terminated at –25 ^{o}C rather than at lower subzero temperatures (Massip et al. 1987). It should be noted that with the mixture of glycerol-sucrose, seeding is initiated at –7.5 ^{0}C because the freezing point is lower than with the glycerol alone. Seeding is considered to be essential for embryonic survival this step has to be executed carefully.

Summarizing briefly the described method, embryos are exposed to the CP at around +20°C, cooled to -7°C and seeded to induce ice formation. Then, they are cooled at about 0.3° to 0.5 °C/min to an intermediate temperature of -30° to -35°C before they are plunged into LN_2 for storage. For use, the frozen samples are warmed relatively rapidly at around 300 °C/min. Figure 3. shows the time-temperature profile for Willadsen's method.

As summarized by Rall (2001) in a recent review, embryos of 22 mammalian species have been successfully cryopreserved by this method.

5.2. Rapid / ultra-rapid freezing

Several rapid freezing methods have been described in which pre-implantation embryos can be plunged directly from around 0°C into liquid nitrogen or liquid nitrogen vapour. Rapid freezing method of cryopreservation includes a short equilibration of embryos or oocytes with the cryoprotective agents, followed by holding the straws at -5°C - -30°C for 25-30 min (Bui-Xuan-Nguyen, 1984; Chupin, 1986, 1987) or placing them in the vapour of liquid nitrogen for one or two min before plunging into LN_2. It is simple, less time consuming than other methods and does not require seeding of the extracellular medium or elaborate and costly freezing units. It was attempted for the first time, with mouse embryos by Wood and Farrant (1980). Miyamoto and Ishibashi (1986), Takahashi and Kanagawa (1990) and Cseh et al. (1997) used liquid nitrogen vapour for the rapid freezing of mouse embryos, stating that slow cooling rates are unnecessary prior to freezing and survival rates are excellent at appropriate

concentrations of sucrose and glycerol. This freezing procedure uses lower cryoprotectant concentrations than the vitrification method consequently there are fewer problems associated with cryoprotectant toxicity (Fahy, 1986; Rall, 1987).

The most important parameters being investigated by the researchers are the types and combinations of cryoprotectants and their concentrations, the developmental stage of the embryo and time of pre-exposure plus the degree of dehydration prior to plunging (Cseh et al., 1997).

Partial dehydration or pre-equilibration with a low concentration of cryoprotectant followed by partial permeation of a high concentration of cryoprotectant enables the cells to supercool to –196^{o}C during rapid freezing without the formation of damaging intracellular ice. Using a very short exposure period (45sec), it is not expected that complete permeation of the high concentration of the cryoprotectant takes place. Thus, complete penetration of cryoprotectants is not necessary for the protection of embryos during rapid-freezing (Nowshari and Brem 1998).

The simultaneous dehydration in a nonpermeating agent (sucrose) and permeation of the cell membranes with a permeating agent (ethylene glycol) enables the cells to supercool without the formation of lethal intracellular ice (Cseh et al.,1997).

In the eighties, the combination of glycerol (1.0-4.5M) and sucrose (0.15-1.0M) was most widely used and these first investigations reported that mouse embryos can be cryopreserved effectively by this method (Wood and Farrant, 1980; Takeda et al., 1984, Reichenbach et al., 1988, Xu et al., 1988). It was shown that there is an interaction between glycerol and pre-freeze sucrose concentrations and that elevated concentrations of glycerol required additional sucrose for dehydration (Biery et al., 1986). Researchers suggested that concentrations of glycerol and sucrose above 3.5M and 0.5M respectively, appear to offer no further protection to mouse embryos cryopreserved by rapid freezing. In addition, the combination of 3.5M glycerol and 0.5M sucrose was reported as the optimal concentration in different experiments (Biery et al., 1986).

Recent experiments comparing different cryoprotectants report that the method can be applied using glycerol, propylene glycol and DMSO, but the most successful one is ethylene glycol (all together with sucrose) (Gutierrez et al., 1993). At rapid freezing also, ethylene glycol seems to have a very low toxicity even when used in high concentrations. Moreover, EG penetrates easily inside embryo blastomeres and restricts ice crystal formation. The time required for penetration and removal of the cryoprotectant is longer for glycerol, DMSO and propylene glycol than for EG.

According to Kasai et al. (1981) EG is the least toxic compared to PG and GLY. So the use of EG has two very important advantages over those of other cryoprotectants which are its low toxicity and quick penetrating ability (Cseh et al., 1997).
The developmental stage in which mouse embryos are subjected to quick freezing affects survival and development in vitro (Cseh et al., 1997). There is interaction between the protocol and the developmental stage. In case of Cseh et al. 1997 it was reported that most (80%) morula and early blastocyst stage embryos survive the procedure. Nowshari and Brem (1998) reported that expanded blastocyst stage was the best for their rapid freezing protocol.

5.3. Vitrification

Vitrification is an alternative form of cryopreservation, a simple, rapid technique that requires minimal equipment. It is based on the fact that concentrated solutions of cryoprotectants (vitrification solutions) don't crystallize when cooled to low temperatures but rather become a non-structural solid, a very viscous glass-like state of matter. This glassy state is not only within but also outside the cell and thus preventing the formation of ice crystals during cooling (Vajta et al., 1999). Vitrification, unlike the other methods of cryopreservation, eliminates ice formation. In addition, by passing the embryos rapidly through the dangerous +15 to -5 °C zone the possibility of chilling injury is reduced (Dobrinsky, 1996; Martino et al., 1996; Isachenko et al, 1998; Zeron et al., 1999). The disadvantages of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation, like toxic, osmotic and other injuries, and the reduced control on the level of dehydration of the cells.
The general outline of the technique is as follows:

a) equilibration of the embryos in diluted vitrification solution at 4 or 20ºC for usually 10 min,

b) transfer to a more concentrated vitrification solution and

c) direct plunging into liquid nitrogen.

The most important variables in vitrification are the speed of freezing, the concentration of the cryoprotectants (the critical concentration that is required for vitrification), sample size and carrier systems. The cooling rate is very high as well as the thawing

rate. (At a cooling rate of approximately 107 °C / s even pure water vitrifies; Rall, 1987). The maximal cooling rate that can be achieved by plunging the commercial 0.25 ml sealed straw to LN_2 is around 2500 °C / min. This cooling rate requires 5 to 7 M concentration of cryoprotectant solution, which is several orders higher than what is needed for traditional equilibrium freezing.

Rall and Fahy in 1985 first reported vitrification as a new technique for cryopreservation of embryos using a mixture of four cryoprotectants. In 1986 Massip et al. developed the technique and achieved 53.8% success in vivo with bovine embryos. The vitrification solution consisted of 25% glycerol and 25% propylene glycol. In 1987 Rall reported good results with 6.5M glycerol as vitrification solution. Later in 1992 Ishimori et al. compared 6 different vitrification solutions and concluded that glycerol and DMSO as well as ethylene glycol and DMSO were successful, with EG and DMSO being successful for both morulae and blastocyst stage embryos. In 1999 Cseh et al. compared the efficiency of two vitrification solutions namely RALL (1987) and MASSIP (1986) in mouse embryos of different developmental stage. They concluded that the compacted morulae seem to be the most suitable for vitrification and reduction in temperature is not required. They also stated that the survival rate of expanded blastocysts can be increased up to 10-fold using MASSIP solution and equilibration at 4ºC. Horlacher and Brem (1994) solidified previous conclusions by reporting that pre-cooling of the vitrification medium gives significantly higher results for the MASSIP method and that there is developmental stage-dependent success using the MASSIP or RALL method, whereas the ISHIMORI (1992) method (EG and DSMO) is suitable for both, compacted morulae and blastocysts.

The improvement of this technique lies in increasing further the cooling and warming rates, through which the cryoprotectant concentration and toxicity and the chilling injury can be reduced. Cells, samples being immersed in liquid nitrogen induce extensive boiling, consequently a vapour coat forms around the cells or samples. As a result, the vapour can create effective insulation that cuts down temperature transfer resulting in the decrease of the cooling rate (Liebermann et al., 2002). To achieve increased cooling rates, direct contact is needed between the cryoprotectant solution and the liquid nitrogen or/and the volume of the vitrification solution shall be reduced (this also prevents heterogeneous ice formation (Rall, 1987)). Nearly all the new techniques are based on these principles. Landa and Tepla (1990) simply dropped the mouse embryo directly into liquid nitrogen. This method can be named as “microdrop

method", and was used also successfully for bovine embryos and oocytes (Riha et al., 1991; Yang and Leibo, 1999; Papis et al., 1999). This scheme was improved by Steponkus et al. (1990) and utilized for bovine embryos by Martino et al. (1996), Arav and Zeron (1997). The improved method contains the placing the embryos on electron-microscope grid before submerging into liquid nitrogen. Vajta et al. published a novel method in 1997. In his Open Pulled Straw technology the carrier of the embryos is a narrow plastic tube and the approximate volume of 1 µl cryoprotectant. This method was employed successfully in numerous trials (Lewis et al., 1999; Vajta et al., 1999; Chen et al., 2000; Lopez-Bejar and Lopez-Gatius 2002). An alternative to OPS is the glass micropipette technique (GMP), used fruitfully by Kong et al. (2000). The GMP vessel permits higher freezing and warming rates than OPS due to the higher heat conductivity of the glass and lower mass of the solution containing the embryos. Other minimum volume cryopreservation techniques have been developed such as the cryoloop or cryotop technique and have been used successfully with several species and developmental stages (Hochi et al., 2004; Lane et al., 1999). Another novel method of vitrification is the so-called solid surface vitrification, recently being investigated de novo by Dinnyes. This method uses a metal surface cooled to the temperature of liquid nitrogen (Dinnyes, 2000; Bagis et al., 2002, 2004).

Increasing the hydrostatic pressure of the vitrification solution has a theoretical inportance that facilitates vitrification by elevating the glass transition temperature (discussed later).

5.4. A brief summary to the thawing of frozen embryos

Thawing is the re-warming of the frozen embryos. With thawing, water osmotically enters the dehydrated embryos until an osmotic equilibrium is reached between the intracellular and extracellular compartment. During the thawing process injuries might occur to the embryos as previously stated and for this reason thawing protocols are developed.

The optimum thawing rate depends on the cryopreservation technique. When embryos are plunged into liquid nitrogen between –30 and –40 °C following conventional freezing, rapid thawing is essential to prevent residual water in the cells re-crystallising during warming. As a thawing rate, 250-500 °C/min is applicable, which

can be achieved by placing the frozen sample to 25-37 °C water bath. If the thawing rate was slow, the small-sized ice crystals formed by the minimal amount of residual water inside the cell have the time to re-crystallize and form larger ice crystals, which are detrimental to the cell (Whittingham, 1979; Willadsen, 1977).

When embryos are plunged into liquid nitrogen below - 60 °C following conventional freezing, slow thawing is essential to provide the cells enough time for rehidration during warming. As a thawing rate, 4-25 °C / min is applicable. During this freezing process embryos dehydrate; residual water can not be found inside the cell. The sufficient influx of water that promotes the rehidration of the cell and prevents osmotic damage can only be achieved by slow thawing (Mauer, 1978; Whittingham, 1979; Mazur, 1980).

6. HYDROSTATIC PRESSURE EFFECTS ON BIOLOGICAL SYSTEMS

Introducing hydrostatic pressure in the practical process of embryo-cryopreservation is a new idea, though Fahy et al. (1984) mentioned the possible use of considerably increased hydrostatic pressure as an additional factor that may facilitate vitrification, but also considered that it had few practical consequences in reproductive biology.
Our attention turned towards the possible benefits of the use of high hydrostatic pressure (HHP) in embryo-cryopreservation based on two basically different phenomena:

1) Solutions can be maintained unfrozen even at low subzero temperatures by applying a certain pressure to them (Bridgman, 1911; Bridgeman, 1970): the phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level.
Studies have been published about the application of this idea in the subzero preservations of organs to be transplanted (Takahashi et al. 2000, 2001); but we conducted experiments in this subject for the first time with embryos.

2) Amongst the several influences of HHP on biological systems, its effects on the cells` proteins gave us the idea to put effort in this subject. High hydrostatic pressure was reported to induce the production of “shock proteins” in bacteria, which can provide a possibility of cross-protection to other environmental stresses (Wemekamp-Kamphuis, 2002; Welch, 1993). Food microbiologists study this phenomenon with food-pathogen bacteria (Ritz et al., 2002; Spilimbergo et al., 2002; Butz et al., 1986); but we conducted experiments in this subject for the first time with embryos.

6.1. *Physical aspects of hydrostatic pressure at freezing*

Since 1912 it has been known that water undergoes different phases when submitted to hydrostatic pressure at different temperatures (Bridgman, 1911). As shown in Figure 4., the phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level (Bridgeman, 1970).

Kanno et al (1975) concluded that the supercooling limit of water, which is -38 °C at room temperature, is lowered by initial increase of pressure, reaching a minimum value of -92 °C at 200 MPa.

MacFarlane et al. (1986, 1987) demonstrated that the glass transition temperature of the solutions rises with increased pressure. For example a solution of 35 % (w/w) DMSO at normal atmospheric pressure cannot be supercooled below -80 °C; however the freezing point is rapidly depressed with increasing pressure up to 130 MPa, at which the solution becomes glass forming (MacFarlane, 1981).

A recent study by Routray et al. (2002) showed that hydrostatic pressure (5 MPa) facilitated the uptake of DMSO in the experiment conducted with eggs and embryos of medaka (*Oryzias latipes*), though there was a rapid loss in the viability.

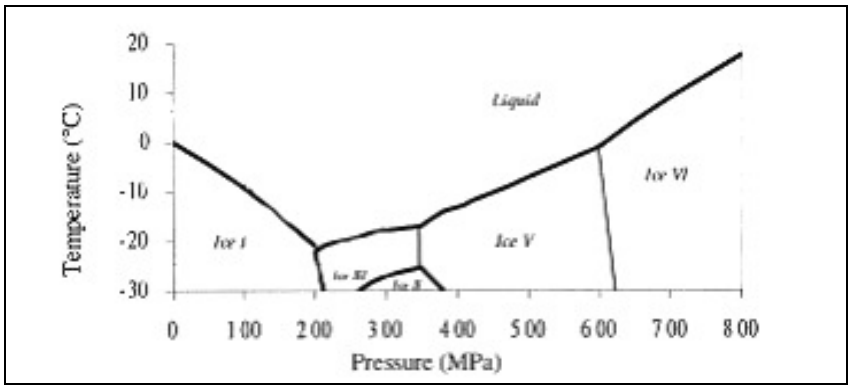


Figure 4. Phase transitions of water at different pressures.

Atmospheric pressure ice (known as ice-I) is less dense than water. Above about 250 MPa pressure ice does not have the same crystal structure as it has at lower pressures. In fact, there are at least 13 other crystal forms.

Different crystal structures result from the application of high pressures causing increasing hydrogen-bond deformation. In the very dense crystals ice-VII and ice-VIII,

each oxygen atom is surrounded by eight nearest neighbours (coordination number is 8) at 0.286nm (in contrast to four nearest neighbours at 0.275nm in ice-I). The hydrogen bonds are lengthened in these dense ice crystals, but each oxygen atom remains hydrogen-bonded to only four hydrogens. Ices II, VIII and IX cannot be obtained directly from liquid water, but can only be obtained by cooling or decompressing ices III, V, VI or VII. Cubic ice (ice-Ic) can be formed by cooling ices II, III, and V to liquid nitrogen temperature and then warming. Ice II can be brought to liquid nitrogen temperature with no changes in structure (Kalichevsky, 1995).

6.2. Biological aspects of hydrostatic pressure

Hydrostatic pressure, as an important environmental factor, has immense effects on the biological functions and viability of living organisms. For this reason, many fields of science are currently focussing on it in order to research their adaptation mechanisms (biologists observe piezophilies and mesophilies (Castellini, al., 2002; Macdonald, 1997), microbiologists study pressure effects for high pressure inactivation of food-pathogen microorganisms (Ritz, et al., 2002; Spilimbergo et al., 2002; Butz and Ludwig, 1986)). Studies show that hydrostatic pressure can damage biological membranes (Macdonald, 1987; Schuster and Sleytr, 2002; Tauc et al., 2002; Aldridge and Bruner, 1985; Yager and Chang, 1983; Péqueux and Gilles, 1978), proteins (Schmid, et al., 1975; Sonoike, et al., 1993; Silva, et al., 2001; Gross and Jaenicke, 1994; Jaenicke, 1991; Weber and Drickamer, 1983), RNA and DNA (Murakami and Zimmerman, 1973), and alter the cells' metabolism (Ernest, et al. 1966; Abe, et al., 1999). After a certain time the changes are irreversible and lethal for the cells, but the barotolerance of some pressurized organisms can be improved (Noma and Hayakawa, 2003; Seki and Toyoshima, 1998).

Hydrostatic pressure in the range of 30-50 MPa usually inhibits the growth of various organisms: the initiation of DNA replication is one of the most pressure-sensitive intracellular processes (Abe, et al., 1999). Uncharged ribosomes (ribosomes without mRNA or tRNA) become dissociated at pressures >60 MPa, which also limits cell growth under conditions of high hydrostatic pressure. The effects vary in severity depending upon the magnitude and duration of compression (Weber and Drickamer, 1983).

The cell membrane is noted as a primary site of pressure damage (Palou et al., 1997). High hydrostatic pressure treatments can alter the membrane functionality such as active transport or passive permeability and therefore perturb the physico-chemical balance of the cell. The physical state of the lipids that surround membrane proteins plays a crucial role in the activity of membrane-bound enzymes and there is considerable evidence that pressure tends to loosen the contact between attached enzymes and membrane surfaces, as a consequence of the changes in the physical state of lipids that control enzyme activity.

The application of pressure can lead to a population of conformers of proteins, including partially or completely unfolded conformations. Pressure can cause the denaturation of proteins by the combined effects of breakage of intraprotein interactions and release of cavities followed by the binding of water. Reports state that hydrostatic pressure also enhances the production of shock proteins (Wemekamp-Kamphuis, et al., 2002; Welch, et al., 1993). Recent studies describe, that instabilities caused by sublethal cold shock in the normal protein synthesis in bacteria are overcome by the synthesis of so-called cold-shock proteins (CSPs) (Phadtare et al., 1999). CSPs are suspected to have many functions such as RNA chaperones (Graumann and Marahiel, 1999) or transcription activators (LaTena et al., 1991); it is assumed, that they also play a role in the protection against freezing (Wouters et al., 1999). Further investigations found, that the production of CSPs is not only induced by cold shock, but by other environmental stresses also. In E. coli, for example, a type of CSP is produced by nutritional stress (Yamanaka et al., 1998). Another trial showed that high hydrostatic pressure treatment provoked the production of certain cold-induced proteins and heat shock proteins (Welch et al., 1993). Since cold-shock and high pressure-treatment both increases CSP levels, trials were conducted about the possibility of cross-protection. Wemekamp-Kamphuis et al. (2002) found, that the level of survival after pressurization of cold-shocked Listeria monocytogenes was 100-fold higher than that of the cells growing at 37 °C. More and more attention is paid recently to study the role of shock proteins in cryopreservation. Huang et al. (1999) published, that a substantial decrease of a shock protein, HSP90, might be associated with a decline in sperm motility during cooling of boar spermatozoa. Wen-Lei et al. (2003) reported that HSP90 in human spermatozoa was decreased substantially after cryopreservation that may result from protein degradation. Derzelle et al. (2003) have investigated the effect of overproducing each of the three cold shock proteins (CspL, CspP, and CspC) in the mesophilic lactic acid

bacterium Lactobacillus plantarum NC8. CspL overproduction transiently alleviated the reduction in growth rate triggered by exposing exponentially growing cells to cold shock, suggesting that CspL is involved in cold adaptation. The strain overproducing CspC resumed growth more rapidly. They also found, that overproduction of CspP led to an enhanced capacity to survive freezing. A Hungarian team reviwed the structure, function and clinical application of HSPs (Csermely et al., 1998).

The effects of pressure on metabolic features in living organisms are thought to be very complex. As a basic rule, the physical or biochemical processes at altered pressure conditions are governed by the principle of Le Chatelier. The principle maintains that pressure favours reactions, which result in a decrease in volume and inhibits reactions with an increase in volume (Palou et al., 1997). All reactions that are accompanied by a volume decrease speed up considerably. Elevated hydrostatic pressure tends to shift equilibrium towards the production of ionized species. The release of protons from weak acids such as H_2PO_4 or H_2CO_3 is enhanced, the hydration and ionization of CO_2 (HCO_3^- and H^+) are facilitated which potentially causes intracellular acidification (Abe and Horikoshi, 1995, 1997, 1998). Pressure effects on microbial ATPase can disturb the proton efflux from the cell interior, also causing internal acidification (Murakami and Zimmerman, 1973; Welch et al., 1993).

The accumulation of the pressure effects is lethal beyond a certain level: while irreversible changes of certain biomolecules take place at higher pressures, at 300 MPa most bacteria and multicellular organisms die. However, tardigrades -in their active state they die between 100-200 MPa- can survive up to 600 MPa if they are in a dehydrated `tun` state (Seki and Toyoshima, 1998). Barotolerance of some bacteria can also be increased through adaptation to low temperature during pre-incubation (Noma and Hayakawa, 2003). The level of survival of pressurized Listeria monocitogenes strikingly increased 100 fold after a previous cold-shock (Wemekamp-Kamphuis et al., 2002).

While food-microbiologists study the above mentioned processes in order to kill detrimental microorganisms, the aim of our studies was to lay down the basic features of pressure-treated embryos and to try to prove its use –through the alleged principle of cross-protection- in embryo-cryopreservation in order to improve survival.

III. OWN EXPERIMENTS

1. SURVIVAL OF RAPIDLY FROZEN HATCHED MOUSE BLASTOCYSTS

1.1. *Abstract*

The objective of the present study was to examine the effect of rapid freezing on the *in vitro* and *in vivo* survival of zona-pellucida-free hatched mouse blastocysts. Hatched blastocysts were rapidly frozen in a freezing medium containing either ethylene glycol or glycerol in 1.5 M or 3 M concentration. Prior to freezing, embryos were equilibrated in the freezing medium for 2 min, 10 min, 20 min or 30 min at room temperature. To freeze them, embryos were held in liquid nitrogen vapour (approximately 1 cm above the surface of the liquid nitrogen (LN_2)) for 2 min and then immersed into LN_2. After thawing, embryos were transferred either to rehydration medium (DPBS + 10% foetal calf serum + 0.5 M sucrose) for 10 min or rehydrated directly in DPBS supplemented with foetal calf serum. *In vitro* survival of embryos frozen with EG was higher than those frozen with GLY. The highest survival was obtained with 3 M EG and 2 min or 10 min equilibration prior to freezing, combined with direct rehydration after thawing. Frozen blastocysts developed into normal foetuses as well as unfrozen control ones did, with averages of 30% (control), 26% (EG) and 15% (GLY). The results show that hatching and hatched mouse blastocysts can be cryopreserved by a simple rapid freezing protocol in EG without significant loss of viability. Our data indicate that the mechanical protection of the zona pellucida is not needed during freezing in these stages.

1.2. Introduction

The first report on successful cryopreservation of embryos was published more than 30 years ago (Whittingham, 1971). Since then, the attention of cryobiologists has been mostly focused on freezing of oocytes and zona intact embryos. The culture conditions are optimised for non-hatched embryos with intact ZP. Moreover, the presence of ZP is required in the breeding animal embryo trade in order to minimize the risk of disease transmission. Thus, the freezing protocols are designed and investigated accordingly. Although, at the pioneer studies the first calf (Wilmut and Rowson, 1973), lamb (Willadsen et al., 1976) and piglets (Kashiwazaki et al., 1991) from frozen embryos were produced from hatched blastocysts.

Recently, more attention is paid to the cryobiology of embryos being in later developmental stages such as hatching and hatched blastocyst. In human in vitro fertilization (IVF) to lower the incidence of multiple gestations and improve implantation rates, the embryos are cultured to the blastocyst stage, and transferred at the hatching or hatched stages (Khorram et al., 2000; Gardner et al., 1998; Yoon et al., 2001). Moreover, it is known, that cryopreserved embryos undergo physicochemical changes that could inhibit hatching, thus contributing to unsuccessful conceiving (Ouhibi et al., 2000). Consequently in the last years the interest is increasing in freezing of hatched blastocysts.

In human IVF, a few reports have been published recently on successful transfer of frozen-thawed hatched blastocysts using propanediol-sucrose method (Quintans et al., 2001). Shaw et al. (1995) examined the survival of mouse expanded and hatched blastocysts after conventional freezing with propanediol and ethylene glycol using different thawing procedures. Expanded and hatched blastocysts were treated together in the same groups with different freezing/thawing protocols. Results showed that mouse blastocysts being in late developmental stages can be successfully cryopreserved by conventional slow freezing, but no separate experiment was carried out on zona free embryos.

In another study, Shaw et al. (1991) used simple rapid freezing for cryopreserving different praeimplantation stage mouse embryos including hatching blastocysts with 4.5 M DMSO and found, that the developmental stage of embryo at the time of freezing and

equilibration time influenced the post-thaw survival in vitro and in vivo. The in vitro survival of hatching blastocysts equilibrated at 0 °C for 3 or 6 min and 10 to 60 min was 40 % and between 40-60 %, respectively. The 3 min equilibration at RT resulted in 20 % survival. The ratio of the normal foetuses at 15 days autopsy was 52 %.

The most complex experiment on the cryopreservation of hatched mouse blastocysts was performed by Zhu et al. (1996). Embryos were equilibrated for 1.5 or 2 min in the one-step, or 5-10 and 0.5 min in the two-step method and then vitrified in two vitrification solutions containing EG or GLY, Ficoll and sucrose. The highest in vitro survival rate (65%) with EG was obtained at the one-step method with embryos that hatched earlier. The results were better with GLY (89-94%), obtained by two-step method. The highest in vivo survival rate (40-44%) was achieved with embryos transferred to recipients on Day 3 or Day 4 of pseudopregnancy.

The objective of our study was to examine the effect of rapid freezing with EG or GLY on the in vitro and in vivo survival of zona-free mouse blastocysts. The influence of different equilibration time prior to freezing and re-hydration methods after thawing was also studied.

1.3. Materials and methods

1.3.1. Animals

CB6F1 (Charles River, Germany) mice were housed under standard conditions (22 +/- 2°C; 12 h dark / 12 h light; water and food ad libitum).

1.3.2. Embryo production

Females were superovulated by intraperitoneal injection of 10 IU of PMSG (Sigma, USA) followed by 10 IU of hCG (Sigma, USA) 46 h later. After 6 hours of hCG administration, females were mated with fertile males in monogamous pairs. One to two cell stage embryos (Day 0 and Day 1) were harvested by flushing the oviduct with FertiCult Flushing medium (FertiPro N.V., Belgium). Embryos were cultured at 37 ^{o}C in thermostat with 5 % CO_2 and maximal humidity in air. Embryos, between one-cell and compact morula stages, were cultured in G 1.2 medium (Vitrolife, Göteborg) under Ovoil (Vitrolife, Göteborg). Then, the embryos were transferred and cultured in G 2.2 (Vitrolife, Göteborg) under Ovoil until the hatching/hatched blastocyst stage.

1.3.3. Freezing and thawing of embryos

Hatching and/or hatched blastocysts on Day 5 (140 -142 h after hCG injection; Day 0 = plug formation) were randomly allocated into different treatment groups, including control group. Embryos were equilibrated in DPBS + 10 % FCS + 0.25 M sucrose containing either 1.5 or 3.0 M EG (Group I.) or 1.5 or 3.0 M glycerol (Group II) for 2, 10, 20 and 30 min at RT. Then, the embryos were loaded in freeze medium into 0.25 ml straws (7-8 embryos/straw) and held 1 cm above the surface of liquid nitrogen in LN_2 vapour for 2 min. Finally, the embryos were immersed into LN_2 (Cseh et al., 1997; Takahashy and Kanagawa, 1990).

For embryo thawing, the straws were air-thawed for 20 seconds, and then dipped into 22-24 ^{o}C water bath for 2 min. After thawing, the embryos were transferred from the freeze medium to re-hydration medium (DPBS + 10 % FCS + 0.5 M sucrose). After 10 min of re-hydration, embryos were rinsed three times in DPBS supplemented with 10 % FCS (Washing Medium) at RT. Half of the embryos were re-hydrated directly in WM (10 min) at RT. Finally, the embryos were transferred and cultured in G2.2, as above, for 24 hours.

1.3.4. Assessment of in vitro and in vivo survival

Embryo quality was examined just after thawing, 12 h and 24 h later. The embryo survival was evaluated upon morphological appearance: integrity of the blastomeres, re-expansion of the blastocoel, and intactness of the blastomeres were the exact signs of survival. Untreated hatched blastocysts kept in DPBS for 40 min in RT were used as controls.

For in vivo evaluation, frozen/thawed embryos were cultured in G 2.2 for 1-2 hours with 5% CO_2 and maximal humidity in air. Then, the embryos (7-12 embryos per animal) with re-expanded blastocoel were transferred to Day 3 pseudopregnant recipients. Untreated hatched blastocysts were transferred as controls. The recipients were exterminated at 18-19 days of pregnancy.

1.3.5. Statistical analysis

Analysis was performed by ANOVA, when it was inappropriate, by logistic regression; the survival rates were compared to control by chi-square test.

1.4. Results

In vitro and in vivo results of the experiments with EG are summarized in Table 1. A total of 457 embryos rapidly frozen with EG were recovered after thawing. Embryos frozen in 1.5 M EG showed significantly reduced survival compared to the control ones (57% and 43% vs. 97%; $p<0.01$) and to the embryos frozen with 3M EG (57% and 43% vs. 94%, 84%, 85%, 95% and 98%; $p<0.01$). There was no significant difference amongst the groups where the concentration of the cryoprotectant was 3M and 1.5M. The equilibration and rehidration times only showed significant effect on the survival in combination with the concentration of the cryoprotectant, but not on their own. No significant difference was noticed between the survival rates at thawing, and 12 and 24 hours after thawing at each of the groups.

230 embryos were transferred to 25 recipients, 17 of which became pregnant. At the in vivo studies no groups showed significant difference from the control.

Table 1.: In vitro and in vivo survival of zona free mouse blastocysts frozen rapidly in ethylene glycol

				In vitro survival				In vivo survival	
No.	Concentration of the cryoprotectant	Equilibration time	Rehidration time	n	At thawing	12 h after thawing	24 h after thawing	Pregnant recipients / No. of recipients	offspring / transferred +
1	3M	30 min	10 min	66	64 (97%)[a]	62 (94%)[a]	62 (94%)[a]	3 / 4	7 / 27 (26%)
2	3M	20 min	10 min	68	64 (94%)[a]	60 (88%)[a]	57 (84%)**[a]	2 / 4	6 / 23 (26%)
3	3M	10 min	10 min	76	71 (93%)[a]	67 (88%)[a]	65 (85%)**[a]	2 / 3	6 / 19 (31%)
4	3M	2 min	direct	63	62 (98%)[a]	60 (95%)[a]	60 (95%)[a]	2 / 3	6 / 20 (30%)
5	3 M	10 min	direct	58	57 (98%)[a]	57 (98%)[a]	57 (98%)[a]	3 / 4	7 / 27 (26%)
6	1.5 M	2 min	direct	63	38 (60%)*[b]	37 (59%)*[b]	36 (57%)*[b]	3 / 3	7 / 23 (30%)
7	1.5 M	10 min	direct	63	29 (46%)*[b]	27 (43%)*[b]	27 (43%)*[b]	2 / 4	5 / 21 (24%)
	Control	kept in DPBS for 40 min		78	-	76 (97%)	76 (97%)	3 / 3	8 / 27 (30%)

Values marked with * are significantly different from the control group ($P<0.01$) (**: $P<0.05$).

Values marked with [a, b] are different from each other.

+: The numbers of transferred embryos represent the blastocysts implanted into those animals that became pregnant.

In vitro and in vivo results of the experiments with GLY are summarized in Table 2. A total of 503 embryos frozen with GLY were recovered after thawing. The survival in all of the groups was significantly reduced compared to the control ($p<0.01$). The cryoprotectant concentration of 1.5 M proved to reduce significantly the survival rate, while the re-hydration time and equilibration time alone had no significant effect.
At the in vivo studies, 190 embryos were transferred to 21 recipients, 11 became pregnant. No result was significantly different from that of the control group; there was no significant difference within the treated groups, either.

Table 2.: In vitro and in vivo survival of zona free mouse blastocysts frozen rapidly in glicerol.

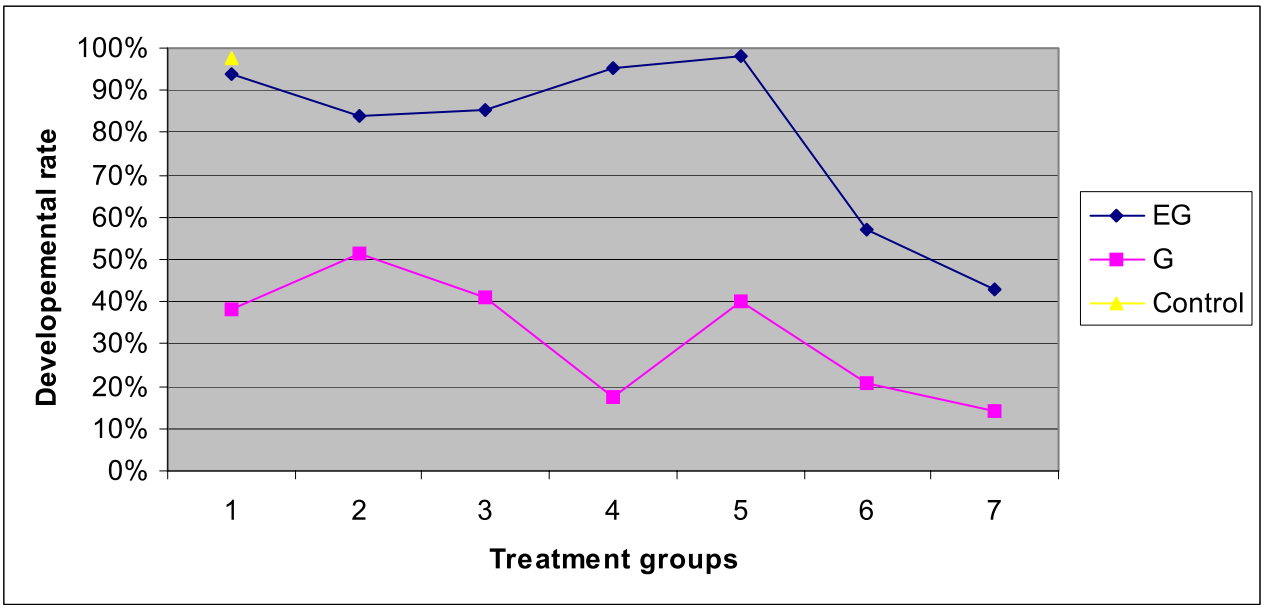
					In vitro survival			In vivo survival	
No.	Concentration of the cryoprotectant	Equilibration time	Rehidration time	N	At thawing	12 h after thawing	24 h after thawing	Pregnant recipients / No. of recipients	offspring / transferred +
1	3 M	30 min	10 min	84	34 (40%)[a]	34 (40%)[a]	32 (38%)[a]	2 / 3	4 / 22 (18%)
2	3 M	20 min	10 min	76	40 (53%)[a]	40 (53%)[a]	39 (51%)[a]	2 / 3	4 / 20 (20%)
3	3 M	10 min	10 min	88	40 (45%)[a]	37 (42%)[a]	36 (41%)[a]	32 / 3	5 / 19 (26%)
4	3 M	2 min	direct	40	9 (23%)[b]	7 (18%)[b]	7 (18%)[b]	0 / 3	(0%)
5	3 M	10 min	direct	80	35 (44%)[a]	34 (43%)[a]	32 (40%)[a]	2 / 3	3 / 25 (12%)
6	1.5 M	2 min	direct	72	54 (75%)[c]	51 (71%)[c]	15 (21%)[b]	1 / 3	1 / 10 (10%)
7	1.5 M	10 min	direct	63	9 (14%)[b]	10 (16%)[b]	9 (14%)[b]	2 / 3	3 / 14 (21%)
	Control	kept in DPBS for 40 min		78	-	76 (97%)	76 (97%)	3 / 3	8 / 27 (30%)

Values marked with [a, b, c] are different from each other.

+: The numbers of transferred embryos represent the blastocysts implanted into those animals that became pregnant.

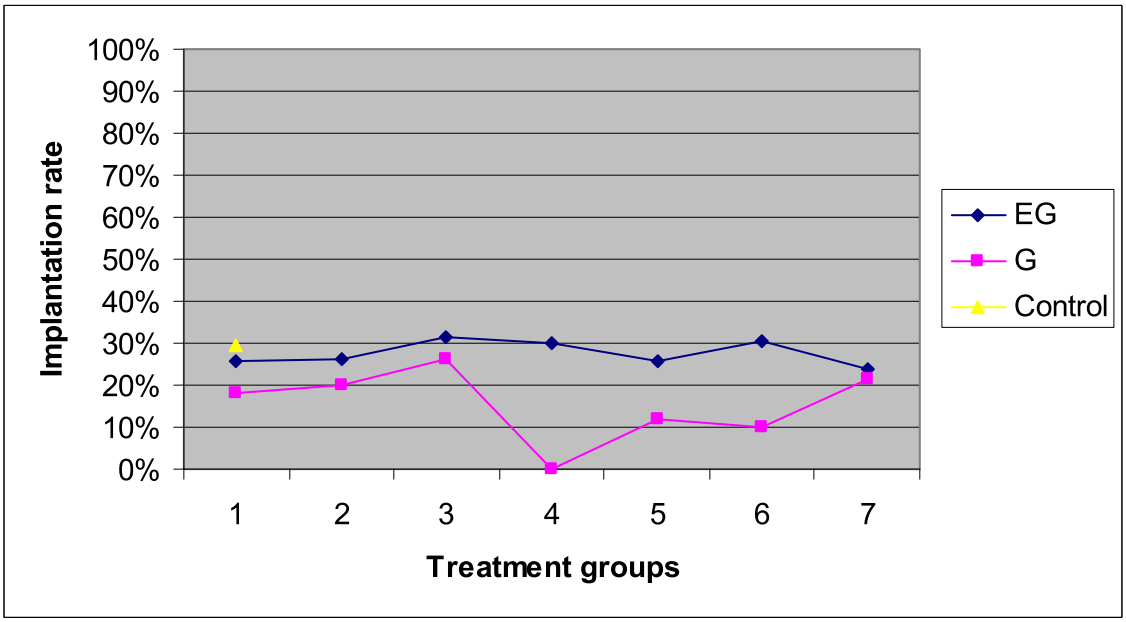
Comparing the in vitro survival rates, there was a significant difference between EG and GLY. Figure 1. shows the differences between the two cryoprotectants at each group.
There was no significant difference between the different groups frozen with EG and GLY in the in vivo survival rates (Figure 2).

Figure 1.: In vitro development (24h) of zona free embryos rapidly frozen with EG or GLY



Numbers on the X axis correspond to the groups in Table 1. and Table 2.

Figure 2. In vivo development of zona free embryos rapidly frozen with EG or GLY



Numbers on the X axis correspond to the groups in Table 1. and Table 2.

1.5. Discussion

Rapid freezing is a simple and effective way for cryopreserving embryos. At quick freezing the concentration of the cryoprotectant is higher than at controlled freezing but lower than the level required for vitrification, thus the toxicity is reduced (Fahy, 1986). The quick freezing method used in this study combines dehydration with non-penetrating sucrose together with the penetrating EG or GLY (Leibo, 1989). In the freezing experiments only blastocysts that hatched early were used, because viability of the delayed embryos is lower, and the freezing might reduce it further.

Comparing the two cryoprotectants, EG proved to be superior to GLY. The best results were obtained with direct re-hydration of embryos cryopreserved in 3M EG (98% and 95%). However these results did not show significant difference from the survival of the other groups cryopreserved in 3M EG. Results indicate, that 2 min equilibration provides enough time for EG to penetrate into the cells, also that the toxicity of EG in 3M concentration is negligible, since the 2 min and 30 min equilibration gave similar survival rates (95% and 94%). The results support the hypothesis that permeability increases as development proceeds (Mazur et al., 1976): EG penetrates into hatched blastocyst faster than into the ones with intact zona. Zhu et al. (1996) found, that 5 min of RT equilibration of hatched blastocysts in 40% EG solution reduced the survival rate to 6%. It may be postulated, that the extremely high concentration of EG penetrating too rapidly into the cells causes more expressed intracellular toxicity then a moderate concentration of 3M. The significant drop in the survival rate when the concentration of EG was reduced to 1.5M (43% and 54%) indicates that the optimal concentration for rapid freezing of hatching/hatched blastocysts in EG is 3M.

Compared to the control group, all groups of embryos frozen with GLY showed a significantly reduced survival. These results are in contrast with the findings of Zhu et al. (1996), who found that vitrification with GLY was more effective than with EG, though the concentration of the cryoprotectant in the vitrification solution was much higher than at rapid freezing. According to our results, the equilibration time did not affect the survival rates, indicating, that the toxicity of GLY might play a similar role in the poor survival rates in each of the groups. But as Zhu et al. (1996) showed that 5 min equilibration in 40% GLY at RT (that resulted in 68% survival vs. 6% for those equilibrated in 40% EG) was less toxic than EG, our results also mean, that the 3M concentration was not optimal for freezing zona-free blastocysts in GLY. The best result

with GLY was obtained with 20 min of equilibration, supports the principle that GLY, due to its higher molecular weight, penetrates slower into the cells.

Rapid freezing of hatched blastocysts with DMSO equilibrated at 0 °C gave similar results as our 3M EG, but not with RT equilibration, where significantly reduced survival was experienced (Shaw et al., 1991).

Transferring hatched blastocysts to Day 3 pseudopregnant recipients, we used 2 days asynchrony, in order to give the embryo more time to resume the normal developmental potential (Zhu et al., 1996; Landa, 1982; Tsunoda et al., 1982). Our results with EG were better than those obtained with GLY, though the differences were not significant. Zhu et al. obtained higher implantation rates with vitrified embryos transferred on Day 3 and Day 4, suggesting that vitrification may be less stressful than freezing (Zhu et al., 1996). Although, the pregnancy results obtained in this study are lower than those ones found in the literature. The poorer pregnancy rates could be explained with technical difficulties connected with housing of the animals and in-experiences of the person doing the transfer.

From the present study, it can be concluded, that zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium, using as short as 2 min equilibration time (that is conveniently enough to load the blastocysts into straws and seal them) at RT. The results also indicate that the mechanical protection of the zona pellucida (ZP) is no longer needed during freezing in these stages.

1.5. References

Cseh, S., Corselli, J., Nehlsen-Cannarella, S.L., Bailey, L.L. and Szalay, A.A. (1997). The effect of quick-freezing in ethylene glycol on morphological survival and in vitro development of mouse embryos frozen at different praeimplantation stages. Theriogenology 48, 43-50.

Fahy, G.M. (1986). The relevance of cryoprotectant `toxicity` to cryobiology. Cryobiology 23, 1-13.

Gardner, D.K., Vella, P., Lane, M., Wagley, L., Schlenker, T. and Schoolcraft, W.B. (1998). Culture and transfer of humane blastocysts increases implantation rates and reduces the need for multiple embryo transfers. Fertility and Sterility 69, 84-88.

Kashiwazaki, N., Ohtani, S., Miyamoto, K. and Ogava S. (1991). Production of normal piglets from hatched blastocysts frozen at -196 C. The Veterinary Record 128, 256-257.

Khorram, O., Saphiro, S.S. and Jones, J.M. (2000). Transfer of non-assisted hatched and hatching human blastocysts after in vitro fertilization. Fertility and Sterility 74, 163-165.

Landa, V. (1982). A simple method for freezing mouse blastocysts. Folia Biologica 28, 266-273.

Leibo, S.P. (1989). Equilibration and non-equilibration cryopreservation of embryos. Theriogenology 31, 85-93.

Mazur, P., Rigopoulos, N., Jackowski, S.C. and Leibo, S.P. (1976). Preliminary estimates of the permeability of mouse ova and early embryos to glycerol. Biophysical Journal 16, 232A.

Ouhibi, N., Sadler F.K., Teramura, D., Eaton, D.L., Larson, J. and Patton P.E. (2000). Blastocyst cryosurvival and pregnancy rate with or without assisted hatching. Fertility and Sterility 74, 52.

Quintans, C.J., Donaldson, M.J., Marazzi, A.N. and Pasqualini, S. (2001). Pregnancy and delivery following the transfer of blastocysts cryopreserved after hatching in propanediol/sucrose. Fertility and Sterility 76, 122.

Shaw, J.M., Diotallevi, L. and Trounson, A.O. (1991). A simple rapid 4.5 M dimethyl-sulfoxide freezing technique for the cryopreservation of one-cell to blastocyst stage praeimplantation mouse embryos. Reproduction Fertility and Development 3, 621-626.

Shaw, J.M., Ward, C. and Trounson, A.O. (1995). Survival of mouse blastocysts slow cooled in propanediol or ethylene glycol is influenced by the thawing procedure, sucrose and antifreeze proteins. Theriogenology 43, 1289-1300.

Takahashy, Y. and Kanagawa, H. (1990). Effect of equilibration period on the viability of frozen-thawed mouse morulae after rapid freezing. Molecular Reproduction and Development 26, 105-110.

Tsunoda, Y., Soma, T. and Sugie, T. (1982). Effect of post-ovulatory age of recipient on survivalof frozen-thawed rabbit morulae. Journal of Reproduction and Fertility 65, 483-487.

Yoon, H.G., Yoon, S.H., Son, W.Y., Im, K.S. and Lim, J.H. (2001). High implantation and pregnancy rates with transfer of human hatching day 6 blastocysts. Fertility and Sterility 75, 832-833.

Whittingham, D.G. (1971). Survival of mouse embryos after freezing and thawing. Nature 233, 125-126.

Willadsen, S.M., Polge, C., Rowson, L.E.A. and Moor, R.M. (1976). Deep freezing of sheep embryos. Journal of Reproduction and Fertility 46, 151-154.

Wilmut, I. and Rowson, L.E.A. (1973). Experiments on the low-temperature preservation of cow embryos. The Veterinary Record 92, 686-690.

Zhu, S.E., Sakurai, T., Edashige, T., Machida, T. and Kasai, M. (1996). Cryopreservation of zona-hatched mouse blastocysts. Journal of Reproduction and Fertility 107, 37-42.

2. MODEL EXPERIMENTS ON PRESERVING MOUSE BLASTOCYSTS BY USING HIGH HIDROSTATIC PRESSURE

2.1. Abstract

Cryoinjuries are almost inevitable during the freezing of embryos. The present study examines the possibility of using high hydrostatic pressure to reduce substantially the freezing point of the embryo holding solution, in order to preserve embryos at subzero temperatures, thus avoiding all the disadvantages of freezing. 210 MPa pressure lowers the phase transition temperature of water to -21 °C. According to our results, embryos can survive in high hydrostatic pressure environment at room temperature; the time embryos spend under pressure without significant loss in their survival could be lengthened by gradual decompression. Pressurization at 0 °C significantly reduced the survival capacity of the embryos; gradual decompression had no beneficial effect to survival at that stage. Based on the present findings, the use of the phenomena is not applicable in this form, since pressure and low temperature together proved lethal for the embryos; though the experiences of this study can be useful applied to other species in different circumstances.

The purpose of the second set of experiments was to study the effect of high hydrostatic pressure treatment prior to freezing to the survival of expanded mouse blastocysts. High hydrostatic pressure has been reported to induce the production of “shock proteins” in bacteria, which can provide a possibility of cross-protection to other environmental stresses. The possible beneficial effects of this alleged principle was examined on embryo freezing: pressure treatment was combined with a cryopreservation protocol. We demonstrated that a preceding pressure-treatment strikingly increases the survival of the frozen blastocysts as well as the speed of resumption of the development, and hatching rate.

2.2. *Introduction*

The process of cryopreservation is harmful to the embryos. Attempts were made to improve survival after freezing: at vitrification highly concentrated aqueous solutions of cryoprotective agents supercool to very low temperatures, allowing intracellular and extracellular vitrification (Rall and Fahy, 1985). Other studies report the use of antifreeze proteins (AFPs) which non-colligatively lower the freezing point of aqueous solutions, block membrane ion channels and thereby confer a degree of protection during cooling. (Baguisi et al., 1987) The toxic effects of the cryoprotectants and the harmful consequences of the osmotic changes are not negligible at any of the described methods.

Since 1912 it has been known that water undergoes different phases when submitted to hydrostatic pressure at different temperatures (Bridgman, 1911). Solutions can be maintained unfrozen even at low subzero temperatures by applying a certain pressure to them (Bridgeman, 1970): the phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level.

High hydrostatic pressure (HHP) was previously used by Takahashi et al. (2000, 2001) at subzero preservation of rat livers for transplantation in order to reduce cryoinjuries.

One aim of the present study was to use high hydrostatic pressure (HHP) in order to reduce substantially the freezing point of the embryo culture medium, thus attempting to preserve embryos –under pressure- at subzero temperature without any of the negative effects of cryopreservation. Though the cryopreservation of mouse embryos is well-established (Shaw et al., 1991, Dinnyes et al., 1995, Rall et al., 2000, Shaw and Kasai, 2001), our experiments can serve as a model in the application of this phenomena to other biological material (embryos of different stages of other species, sperm, etc..).

First we investigated the behaviour of embryos under high pressure environment. We attempted to find a way to improve the survival of pressurized embryos by using gradual decompression, and then we investigated embryo survival when HHP was combined with low temperature.

The physiological mechanisms by which microorganisms adapt to sublethal stresses are not yet understood well. Recent studies describe, that instabilities caused by

sublethal cold shock in the normal protein synthesis in bacteria are overcome by the synthesis of so-called cold-shock proteins (CSPs) (Phadtare et al., 1999). These CSPs are suspected to have many functions such as RNA chaperones (Graumann and Marahiel, 1999) or transcription activators (LaTena et al.,1991); it is assumed that they also play a role in the protection against freezing (Wouters et al.,1999). Further investigations found, that the production of CSPs is induced not only by cold shock, but also by other environmental stresses. In Escherichia coli, for example, a type of CSP is produced by nutritional stress (Yamanaka et al., 1998). Another trial showed that high hydrostatic pressure treatment provoked the production of certain cold-induced proteins and heat shock proteins (Welch et al., 1993). Since both cold-shock and high pressure-treatment increase CSP levels, trials were conducted about the possibility of cross-protection. Wemekamp-Kamphuis et al. (2002) found, that the level of survival after pressurization of cold-shocked Listeria monocytogenes was 100-fold higher than that of the cells growing at 37 °C.

Food-microbiologists study the above mentioned processes in order to kill detrimental microorganisms (Butz and Ludwig, 1986; Wemekamp-Kamphuis et al., 2002; Spilimbergo et al., 2002). However, the objective of our second experiments was to investigate the effect of high hydrostatic pressure on the survival of cryopreserved (vitrified) mouse embryos. The aim of our experiments was to test whether or not high pressure treatment prior to vitrification -through the alleged principle of cross-protection- can improve survival of cryopreserved mouse blastocysts.

2.3. Materials and Methods

2.3.1. Experimental animals and embryo production

CB6F1 (Charles River, Germany) mice were housed under standard conditions (22 +/- 2°C; 12 h dark / 12 h light; water and food ad libitum).

Females were superovulated by intraperitoneal injection of 10 IU of PMSG (Sigma, USA) followed by 10 IU of hCG (Sigma, USA) 46 h later. After 6 hours of hCG administration, females were mated with fertile males in monogamous pairs. One to two cell stage embryos (Day 0 and Day 1) were harvested by flushing the oviduct with FertiCult Flushing medium (FertiPro N.V., Belgium). Embryos were cultured at 37 ^{o}C in thermostat with 5 % CO_2 and maximal humidity in air. Embryos, between one-cell

and compact morula stages, were cultured in G 1.2 medium (Vitrolife, Sweden) under mineral oil, Ovoil (Vitrolife, Sweden). Then, the embryos were transferred and cultured in G 2.2 (Vitrolife, Sweden) under Ovoil until the expanded blastocyst stage. Procedures were approved by the Animal Care and Use Committee of the Faculty.

2.3.2. Pressurization

Blastocysts were loaded into plastic straws without air-bubbles (7-9 embryos / straw), with M2 (Sigma, USA), then straws were heat-sealed. Straws were placed into the pressure-chamber filled with water as pressure medium. The custom-made pressurizing device, which was capable of providing precisely controlled pressure in the range of 1 to 150 MPa was made of stainless steel with the inner diameter of 2 cm, and was connected to a pressure-gauge. Hydrostatic pressure was generated by pushing a piston into the pressure chamber through the manual control of screws. Achieving the desired amount of pressure took from 20 seconds to 5 min (10 MPa to 150 MPa, respectively); the duration of pressure release was 3 seconds. At the experiments where the effects of gradual decompression were investigated, release time was between 30-210 min. At experiments conducted at 0°C, the pressure chamber was simmered in the cooling bath of Bio-cool (FTS-Systems, NY, USA).

2.3.3. Cryopreservation with previous pressurization

Embryos were randomly allocated to three groups. Blastocysts of Group I. were cryopreserved as mentioned below, in a vitrification solution containing 7 M Ethylene glycol (EG) according to Nowshari and Brem (1998). Embryos of Group II. were treated with 60 MPa pressure for 30 min, then were frozen in the same way. Group III. served as untreated control. After thawing, embryos were cultured in vitro for 24 hours.

2.3.4. Cryopreservation

Embryos were equilibrated for 5 min in a solution containing 1.5 M ethylene glycol (EG) (Sigma, USA) and 0.25 M sucrose in M2 (Sigma, USA), supplemented with 10 % Fetal Calf Serum (FCS) (Sigma, USA), then transferred into a vitrification solution (7 M EG, 0.5 M sucrose in M2 with 10 % FCS) pre-loaded in a 0.25 ml plastic straw (7-9 embryos / straw). Finally, straws were heat-sealed. After 1 min exposure to the vitrification solution, the straw was slowly immersed in liquid nitrogen. Straws were thawed by transfer into 30 °C water for 30 sec and then the embryos were recovered and

placed in rehydration medium (0.5M sucrose in M2 supplemented with 10% FCS) for 5 min. Embryos then were cultured in medium G 2.2 as described above (Nowshari and Brem, 1998).

2.3.5. Embryo transfer

Embryos were cultured in G 2.2 for 2 hours as above. Then, they were separated in each experimental group as “dead” and “survived” and were transferred separately (7-12 embryos per animal) to Day 3 pseudopregnant recipients. Untreated blastocysts were transferred as controls.

2.3.6. Evaluation and statistical analysis

Embryo quality was examined just after releasing the pressure or after thawing and after 2, 3, 4, 6, 12, 20 and 24 hours. The embryo survival was evaluated upon morphological appearance: intactness of the blastomeres re-expansion of the blastocoel, and hatching from the zona pellucida were the signs of survival. Untreated blastocysts were used as controls.

For in vivo evaluation, pressurized embryos were cultured in G 2.2 for 2 hours as above. Then 7-12 embryos per animal were transferred into Day 3 pseudopregnant recipients. Untreated blastocysts were transferred as controls. Birth of healthy pups was proof of in vivo survival of the embryos.

The survival rates were compared to control by chi-square test.

2.4. Results

In the first set of experiments embryos were exposed to different hydrostatic pressures from 10 to 150 MPa (by 10 MPa increments) for various times (1s, 5 min, 15 min and 30 min to 300 min by 30min), at room temperature. In each group 14-16 embryos were used and each experiment was repeated 3 times.

The treatment exceeding a certain amount of pressure and time (e.g. embryos challenged by 90 MPa for 30 min or 30 MPa for 3h) caused reversible morphological changes. The expanded blastocysts compacted inside the zona pellucida: the blastocoel disappeared, the size of the blastomeres reduced but their structural integrity showed no alteration. After 4-5 hours of in vitro culture these blastocysts re-expanded and hatched

from the zona pellucida in 24 hours as controls ($p<0.05$) (a). Embryos receiving less impact (e.g. 90 MPa for 1 s or 30 MPa for 2 h) showed no morphological change and hatched within 24 hours of in vitro culture such as controls ($p<0.05$) (b), while embryos challenged with a greater impact (e.g. embryos challenged by 90 MPa for 2 h or 30 MPa for 5 h) did not re-expand from the compacted stage and disintegrated within 2 hours, or were already disintegrated after decompression (c) (Figure 1.).

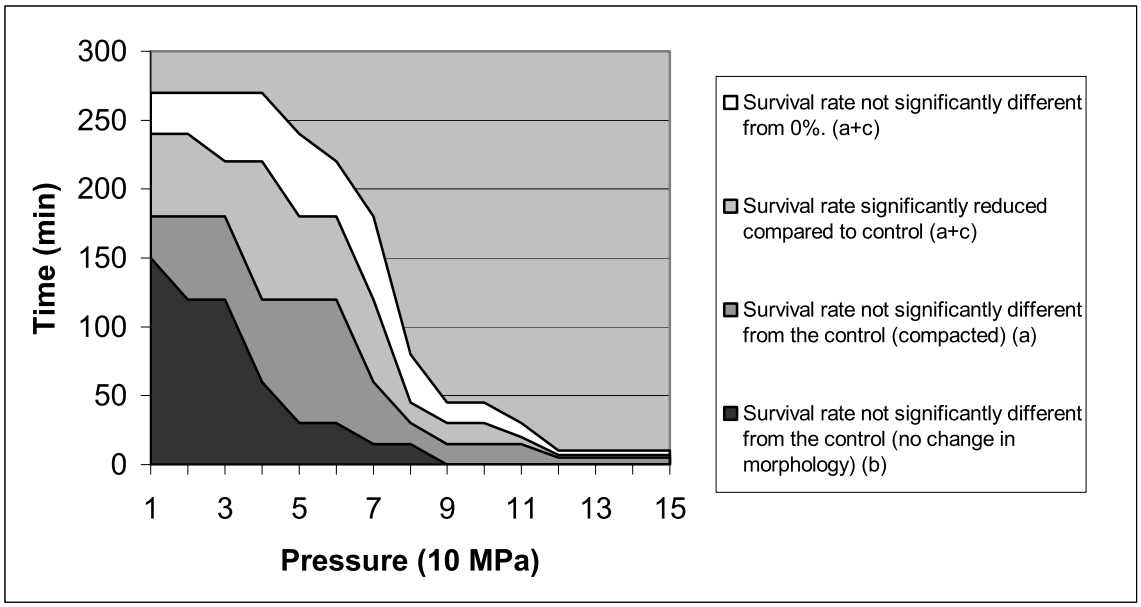


Figure 1. Survival rate of embryos at different pressures between 10 MPa and 150 MPa for different times, between 1 sec to 300 min, at room temperature. 14-16 embryos were used in each group; each experiment was repeated 3 times. The survival and hatching rates of embryos in the fields marked with "a" and "b" are not different from the untreated control ($p<0.05$). The survival rates of the in vitro control groups were between 99 and 97 % (hatching rate: 93-94%) (n=10-12 / group).

The survival rates of the in vitro control groups were between 99 and 97 % (hatching rate: 93-94%) (n=10-12 / group).

For in vivo evaluation, challenged embryos were judged "survived" (a&b) and "dead" (c) after 2 hours of in vitro culture after decompression and were transferred into recipients separately. Out of 170 transferred "a" and "b" embryos, 145 healthy pups were born (85%), but 0 were born from 49 "c" embryos (0%). 36 untreated embryos were transferred as controls, 30 embryos were born (83%).

There were no significant differences between the survival rate, hatching rate (in vitro) and birth rate (in vivo) of the non pressurized control, the compacted and the non-compacted pressurized "a" and "b" embryos ($p<0.05$).

In the second experiment we investigated whether the survival rate of the pressurized embryos could be improved by gradual decompression. Expanded blastocysts were kept at 90 MPa for 30, 60 and 120 min, (where the survival rate at room temperature with instant decompression was 50%, 0% and 0%, respectively) then the pressure was gradually released in 9 steps for 30, 60, 90, 120 and 150 min. The results showed that survival significantly improved by gradual decompression, which had an optimal range depending on the time the embryos had spent under pressure. The optimal come-up time rose the longer the time embryos spent under pressure. The maximal survival rate, achievable by decompression, reduced as the time of the pressurization increased. (Figure 2.)

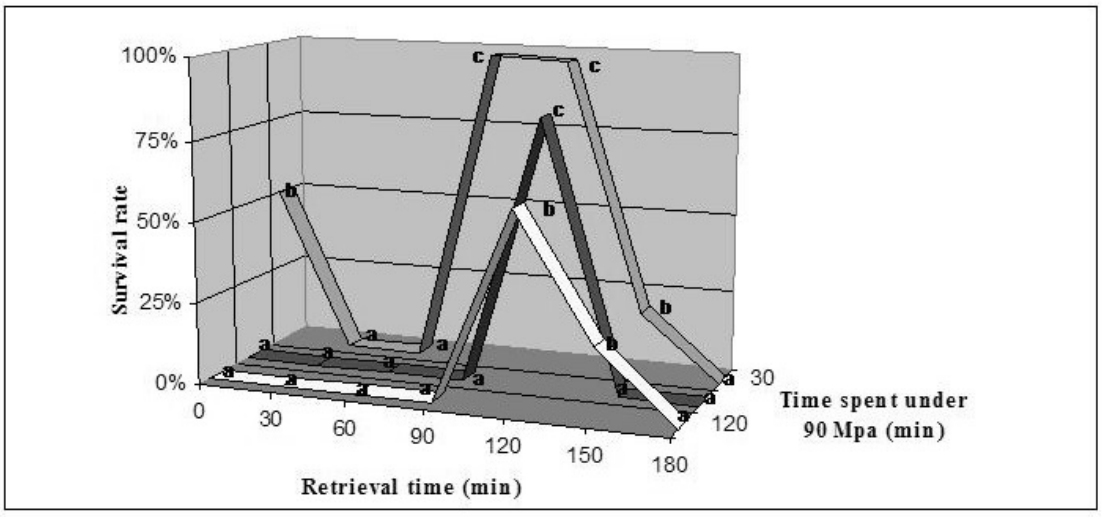


Figure 2. Survival rates of embryos pressurized with 90 MPa for 30, 60, 120 min and decompressed for 30-180 min. (With instant decompression survival at 30, 60 and 120 min was 50%, 0%, 0%, respectively). Survival rates marked on the figure with different superscripts are significantly different from each other ($p<0.05$).

At in vivo evaluation 54 “survived” and 35 “dead” embryos were transferred to 9 recipients. 47 embryos (87%) implanted out of 54 “survived”, and 0 embryos implanted out of the 35 “dead” embryos at the 18 days dissection. The implantation rate of the “survived” group was not different from the control ($p<0.05$).

In the third part of the experiment the role of temperature was investigated on the survival capacity of the pressurized embryos: 30, 60 and 90 MPa pressure was applied to embryos for 1 sec, 5, 10, 15, 30 and 60 min at low temperature (0 °C). While non-pressurized embryos can live at 0 °C for a substantial amount of time without any

significant change in their survival, simultaneous pressure treatment with 30, 60, 90 MPa was lethal for 100 % of the embryos after 45, 10, 5 min, respectively. A significantly reduced survival rate was observed with the embryos pressurized at low temperature compared to the groups treated at room temperature (P<0.01%) (Figures 3a, 3b).

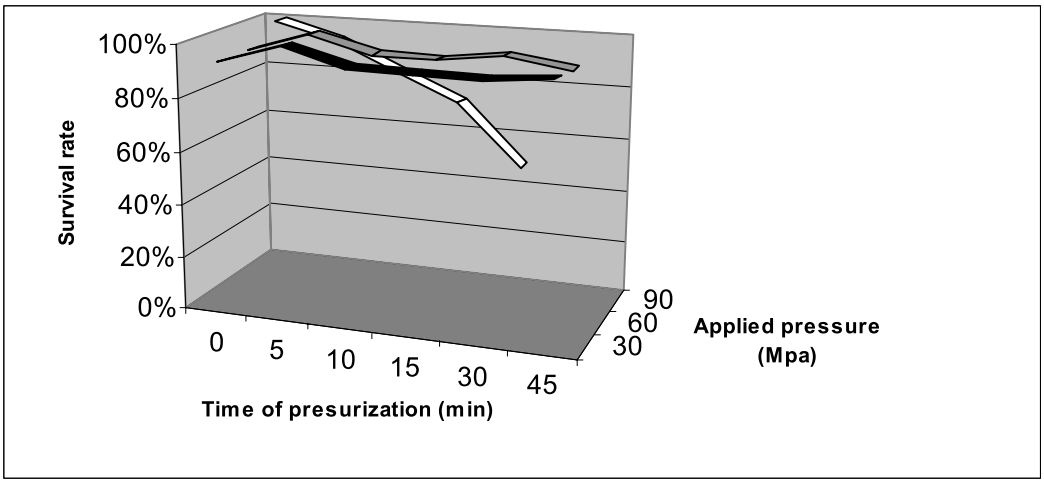


Figure 3a. Survival of embryos pressurized with 30, 60 and 90 MPa for 1 sec to 45 min, at room temperature.

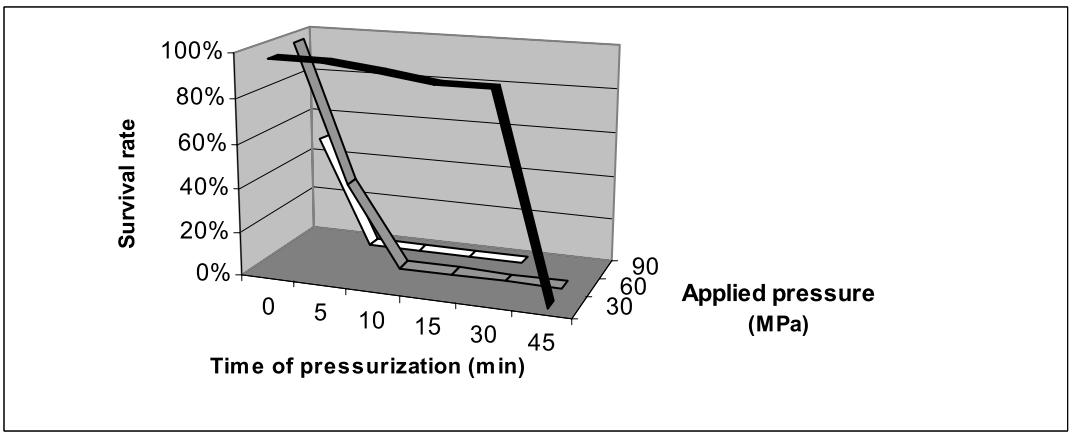


Figure 3b. Survival of embryos pressurized with 30, 60 and 90 MPa for 1 sec to 45 min, at 0 °C. 12-15 embryos were used in each of the groups; each experiment was repeated 3 times. Significant differences are seen between the groups pressurized at room temperature and at 0 °C (p<0.01).

At the in vivo evaluation 40 “survived” and 28 “dead” embryos were transferred to 7 recipients. 34 embryos (85%) implanted out of the 40 “survived”, and 0 embryos implanted out of the 28 “dead” embryos at the 18 days count. The implantation rate of the “survived” group was not different from the control (p<0.05).

Embryos kept at 0 °C under 90 MPa pressure for 30 min were also decompressed gradually. No embryos survived at any of the retrieval times we used (30 60, 90, 120, 150, 180 min). 8-12 embryos were used in each group, experiments were repeated for three times.

In the fourth study we explored whether the survival rate of cryopreserved expanded mouse blastocysts could be improved by pressure treatment before the freezing procedure. 30 -39 embryos were used in each experimental group, experiments were repeated 3 times. Results are presented in Table 1.

Table 1. Survival of frozen-thawed embryos cryopreserved with/without previous pressure treatment

	n	**Signs of survival after 6 hours**		**Signs of survival after 20 hours**			
		½ expanded	Fully expanded	½ expanded	2/3 expanded	Fully expanded	Hatched
Group I. (Frozen without pressure pre-treatment)	115	10 (9%)	**0 (0%)[b]**	20 (17%)	12 (10%)	22 (19%)	**0 (0%)[b]**
Group II. (Frozen with pressure pre-treatment)	95	-	**93 (98 %)[a]**	-	-	3 (3%)	**90 (95%)[a]**
Untreated Control	107	-	**106 (99%)[a]**	-	-	5 (5%)	**101 (94%)[a]**

Letters with different superscript are significantly different from each other ($p<0.01$).

Significant differences were observed in the survival rate between the pressurized and non-pressurized groups ($p<0.01$). The re-expansion was faster (4-6 hours vs. 20 hours) and the survival rate was higher (98% vs. 46%) in those embryos that received pressure treatment before cryopreservation (Table 1.). There was no significant difference between the control and the pressure treated group in the survival and hatching rate.

2.5. Discussion

We explored HHP in the process of cryopreservationon on two separate grounds. As shown in Figure 4., the phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level. Similarly, we reduced the phase transition temperature of the embryo

holding solution through the application of high hydrostatic pressure with the aim to preserve embryos in their culture medium at temperatures below 0 °C, for a substantial period without freezing.

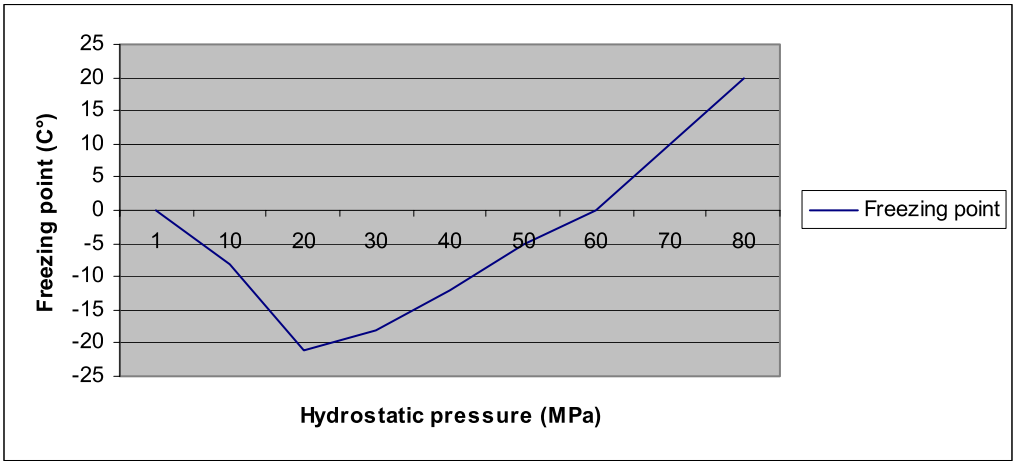


Figure 4. Freezing points of water at different pressures.

Hydrostatic pressure in the range of 30-50 MPa usually inhibits the growth of various organisms: the initiation of DNA replication is one of the most pressure-sensitive intracellular processes (Abe et al., 1999). The effects vary in severity depending upon the magnitude and duration of compression (Murakami and Zimmerman, 1973). The cell membrane is noted as a primary site of pressure damage (Palou et al., 1997). High hydrostatic pressure treatment can alter the membrane functionality such as active transport or passive permeability and therefore perturb the physico-chemical balance of the cell (Yager and Chang, 1983; Aldridge and Bruner, 1985; Macdonald, 1987; Schuster and Sleytr, 2002). The application of pressure can lead to a population of conformers of proteins, including partially or completely unfolded conformations. Pressure can cause the denaturation of proteins by the combined effects of breakage of intraprotein interactions and release of cavities followed by the binding of water (Schmid et al., 1975; Weber and Drickamer, 1983; Jaenicke, 1991; Gross and Jaenicke, 1994; Silva et al., 2001). Recent reports state that hydrostatic pressure enhances the production of shock proteins (Welch et al., 1993; Wemekamp-Kamphuis et al., 2002). The physical or biochemical processes at altered pressure conditions are governed by the principle of Le Chatelier: all reactions that are accompanied by a volume decrease speed up considerably (Murakami and Zimmerman, 1973; Welch et al., 1993; Palou et al., 1997).

The accumulation of the pressure effects is lethal beyond a certain level: while irreversible changes of some biomolecules take place at higher pressures, at 300 MPa most bacteria and multicellular organisms die. However, tardigrades -in their active state they die between 100-200 MPa- can survive up to 600 MPa if they are in a dehydrated `tun` state (Seki and Toyoshima, 1998).
In an early publication (Johnson et al., 1954) the authors showed, that biological systems are able to tolerate high pressures as long as the pressure is reduced slowly.

In order to find out if embryos survive at altered pressure conditions we studied the behaviour of embryos under pressure at room temperature. As highly developed eukaryotic organisms, mouse embryos are more susceptible to the effect of hydrostatic pressure than tardigrades and bacteria. The first set of experiments lays down the basic features of mouse embryos under pressure concerning their morphology and survival. As Figure 1. shows, embryos can survive a substantial amount of pressure without any visible change in their morphology (e.g. 90 MPa for 1 s or 30 MPa for 2 h). As it is shown, embryos compacted depending on the magnitude and the duration of the applied pressure treatment. Since pressure cannot be directly responsible for squeezing the water out of the blastocysts, we assume, based on the cited documents that the compaction of the embryos was due to the consequences of pressure induced production of different proteins (CSPs), reversible alterations in protein structure and metabolic processes. Compacted embryos could regain their normal morphology after 4-5 hours of in vitro culture, and resume development similarly to controls (e.g. embryos challenged by 90 MPa for 30 min or 30 MPa for 3h). The higher the magnitude of the pressure, the less time the embryos survive. Pressure impact exceeding a certain magnitude and duration caused irreversible changes: embryos became disintegrated after 2 hours of in vitro culture or were already disintegrated after decompression (e.g. embryos challenged by 90 MPa for 2 h or 30 MPa for 5 h).

As for the second step, we tried to improve the survival rate of the pressurized embryos by gradual decompression. Our studies showed that the survival rate of the pressurized embryos increased strikingly if they were retrieved gradually. While 60 min at 90 MPa was lethal for all of the embryos, 80 % survived when 120 min gradual decompression was used. A possible explanation of this feature could be that a considerable amount of CO_2 is generated under pressure (Abe and Horikoshi 1995). The hydration and ionization of CO_2 (HCO_3^- and H^+) are facilitated by elevated pressure

because the reaction is accompanied by a decrease in volume (-0.26 ml/mol) in a manner dependent on the magnitude of the pressure applied (Palou et al. 1997, Welch et al. 1993). The intracellularly produced carbon dioxide instantly dissolves then dissociates to give HCO_3^- and H^+, thus also reducing the intracellular pH (Abe and Horikoshi 1995, 1997, 1998, Abe et al. 1999.). We can assume that the equilibrium maintained by elevated pressure is lethal for the embryos at atmospheric pressure. It may be hypothesized that the instant decrease of pressure causes elevated release of CO_2 from its hydrated and ionized form from the cytoplasm, causing immediate death of the embryos. On condition of a certain decompression time, the plasma membrane proteins (H^+-ATPase) (Schmid et al. 1975, Péqueux and Gilles 1978) reversibly inactivated by elevated hydrostatic pressure, start to function again, (together with passive diffusion) shifting the equilibrium gradually towards the physiological state.

Experiments conducted at zero temperature sowed, that the survival of embryos pressurized at 0 °C reduced significantly. While at room temperature embryos had an average survival rate of 90% at 30 MPa for 45 min, none of the embryos survived the same impact at 0 °C. After 10 min or 5 min at 0 °C, none of the embryos survived at 60 MPa and at 90 MPa respectively, though at room temperature the survival rate was around 90 % in both cases.

The higher was the pressure at a low temperature the less time the embryos survived. It can be hypothesized that the metabolism of the embryos plays a more significant role in the adaptation to high hydrostatic pressure as the pressure rises.

Embryos pressurized at 0 °C were also decompressed gradually. Assuming that a slowed metabolism needs more time to restore the normal atmospheric equilibrium we also tried to lengthen the retrieval times, used successfully in the second experiment. The application of gradual decompression at low temperature did not have a beneficial effect on the embryo-survival.

We conclude that gradual decompression of the pressurized embryos significantly increases the survival rate. Pressurization at low temperature (0 °C) significantly reduces the survival capacity of the embryos, and gradual decompression has no effect at this stage. Based on the present experiments the storage of embryos at subzero temperatures under high pressure is not applicable, since the two effects (pressure and low temperature) together significantly reduces survival even at 0°C. These experiments can serve as examples in the application of this phenomena to other biological material.

In the fourth study, our aim was to explore the effect of a simple sublethal shock - hydrostatic pressure- to the survival capacity of the expanded mouse blastocysts to an other "shock": cryopreservation. We explored whether the survival rate of cryopreserved expanded mouse blastocysts could be improved by a certain pressure treatment (a sublethal shock) before the freezing procedure. For the above-mentioned reasons only blastocysts that compacted after pressurization were used in the experiment. We applied a pressure impact of 60 MPa for 30 min – where approximately 90% of the embryos became compacted and survival was not different from the untreated control. According to the results, the applied pressure treatment strikingly improved the in vitro development of the embryos after freezing. After 6 hours 98 % of the pressure treated blastocysts were morphologically (diameter, structural integrity, and general morphology) exactly identical to the control embryos and 95% of the blastocysts fully hatched within 20 hours, together with the controls (Figures 5a & 5b).

Embryos frozen without pressure treatment reexpanded only 20 hours after thawing. The proportion of the re-expanded blastocysts was significantly inferior to those receiving pressure treatment (only 29 % was at least 2/3 expanded). In addition, no embryos hatched from this group.

The survival and hatching rate of embryos frozen without pressure treatment in our experiment were also inferior to the results gained by Nowshari and Brem (1998) with the same freezing protocol, supposedly because of lab conditions (room temperature was 30 °C), but the circumstances of all of our experiments were identical.

We also cryopreserved embryos that were not compacted after the pressurization. The developmental speed, survival and hatching rates were not significantly different from that of the embryos cryopreserved without pressurization.

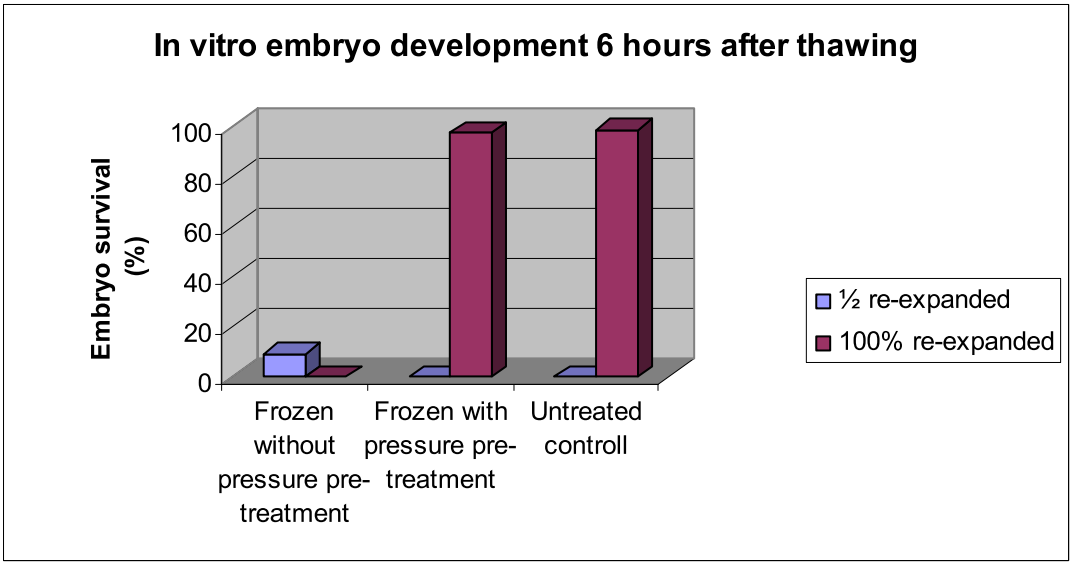


Figure 5a. In vitro development of mouse blastocysts 6 hours after thawing of the groups frozen with or without hydrostatic pressure pre-treatment.

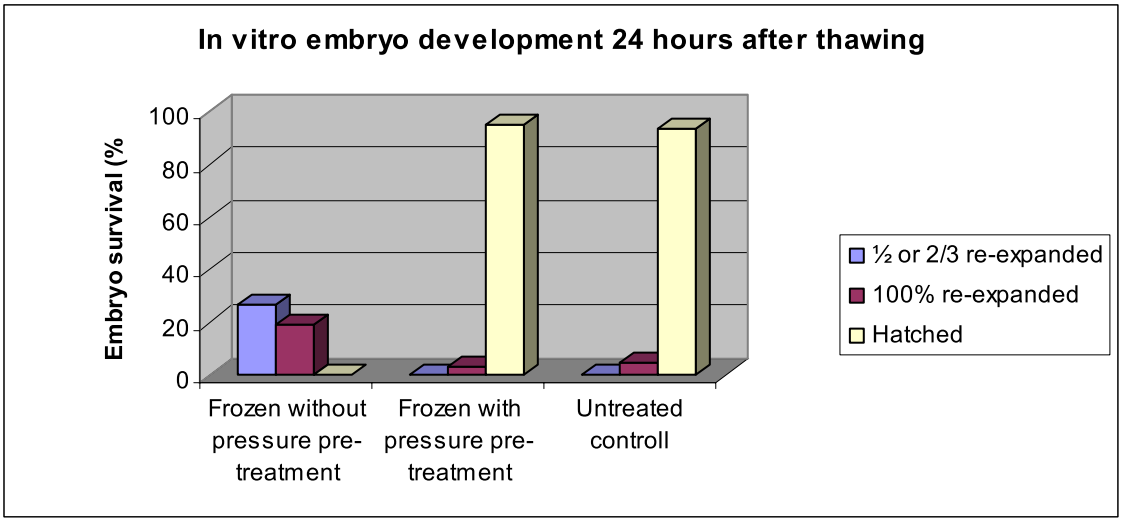


Figure 5b. In vitro development of mouse blastocysts 24 hours after thawing of the groups frozen with or without hydrostatic pressure pre-treatment.

2.6. Conclusion

The cryopreservation of blastocyst stage mouse embryos had been well-established (Shaw et al., 1991, Dinnyes et al., 1995, Nakao et al., 1997, 1998, Rall et al., 2000, Shaw and Kasai, 2001). Our aim was not to invent another protocol for the cryopreservation of mouse embryos, but to put down a model experiment based on

novel bases, which can serve as an example to other cryobiological studies where success rates need to be improved. We showed that embryos can survive a substantial amount of pressure; that the survival of embryos can be improved by gradual decompression, and that 0 °C together with the pressure treatment considerably reduced survival. We also showed, that the applied pressure treatment prior to freezing could improve the in vitro developmental speed, survival and hatching rates of the embryos. Knowledge of this experiment can be useful in improving success rates in embryo-cryopreservation (and embryo-manipulation) of other mammalian species including the human.

Further studies are required to support the deeper understanding of pressure assisted metabolic and structural changes of the pressurized embryos and its role in cross-protection.

2.7. References

Abe, F. and Horikoshi, K. (1995): Hydrostatic pressure promotes the acidification of vacuoles in Saccharomyces cerevisiae. FEMS Microbiol. Lett. 130, 307-312.

Abe, F. and Horikoshi, K. (1997): Vacuolar acidification in Saccharomyces cerevisiae induced by elevated hydrostatic pressure is transient and is mediated by vacuolar H+-ATPase. Extremophiles 1, 89-93.

Abe, F. and Horikoshi, K. (1998): Analysis of intracellular pH in the yeast Saccharomyces cerevisiae under elevated hydrostatic pressure: a study in baro- (piezo) physiology. Extremophiles 2, 223-228.

Abe, F., Kato, C. and Horikoshi, K. (1999): Pressure-regulated metabolism in microorganisms. Trends Microbiol. 7, 447-453.

Aldridge B.E., Bruner L.J., (1985). Pressure effects on mechanisms of charge transport across bilayer membranes. Biochim Biophys Acta 817, 343-354.

Baguisi, A., Arav, A., Crosby, T.F., Roche, J.F. and Boland, M.P. (1987): Hypothermic storage of sheep embryos with antifreeze proteins: development in vitro and in vivo. Theriogenology 48, 1017-1024.

Bridgeman, P.E. (1970): The physics of high pressure. New York: Dover

Bridgman, P.W. (1911): Water in the liquid and five solid forms under pressure. Proceedings of the American Academy of Arts and Science 47, 441–558.

Butz P. and Ludwig H. (1986). Pressure inactivation of microorganisms at moderate temperatures. Physica B+C 139-140, 875-877.

Dinnyes A., Wallace G.A. and Rall W.F. (1995). Effect of genotype on the efficacy of mouse embryo cryopreservation by vitrification or slow freezing methods. Mol Reprod Dev. 40, 429-35.

Graumann P.L. and Marahiel M.A. (1999). Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in Bacillus subtilis. Arch Microbiol 171, 135-138.

Gross M. and Jaenicke R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. Eur J Biochem 221, 617-630.

Jaenicke R. (1991). Protein stability and molecular adaptation to extreme conditions. Eur J Biochem 202, 715-728.

Johnson F.H., Eyring H. and Polissar M.J. (1954). The cinetic basis of molecular biology. Wiley, New York

LaTena A., Brandi A., Falconi M., Spurio R., Pon C.L. and Gualerzi C.O. (1991). Identification of a cold-shock transcriptional enhancer of the Escherichia coli major cold shock gene encoding nucleotide protein H-NS. Proc Natl Acad Sci USA 88, 10907-10911.

Macdonald A.G. (1987). The role of membrane fluidity in complex processes under high pressure. In: Jonnasch H.W., Marquis R.E., Zimmerman A.M., editors. Current Perspectives in High Pressure Biology. London: Academic Press pp. 207-223.

Murakami T.H. and Zimmerman A.M. (1973). DNA syntheseis in Tetrahymena: a pressure study. Cytobios 7, 171-181.

Nakao K., Nakagata N. and Katsumi M. (1997). Simple and efficient vitrification procedure for cryopreservation of mouse embryos. Exp. Anim. 46, 231-234.

Nakao K., Nakagata N. and Katsumi M. (1998). Production of chimeric mice from cryopreserved blastocysts. Exp. Anim. 47, 167-171.

Nowshari M.A. and Brem G. (1998). Effect of cryoprotectants and their concentration on post-thaw survival and development of expanded mouse blastocysts frozen by a simple rapid-freezing procedure. Theriogenology 50, 1001-1013.

Palou E., Lopez-Malo A., Barbosa Canovas G.V., Welti-Chanes J. and Swanson B.G. (1997). Kinetic analysis of Zygosaccharomyces bailii inactivation by high hydrostatic pressure. Lebensm-Wiss U Technol 30, 703-708.

Péqueux, A. and Gilles, R. (1978). Effects of high hydrostatic pressures on the activity of the membrane ATPases of some organs implicated in hydromineral regulation. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **59**, 207-212.

Phadtare S., Alasina J. and Inouye M. (1999). Cold-shock response and cold-shock proteins. Curr Opin Microbiol 2, 175-180.

Rall W.F., Schmidt P.M., Lin X., Brown S.S., Ward A.C. and Hansen C.T. (2000). Factors effecting the efficiency of embryo cryopreservation and rederivation of rat and mouse models. ILAR J. 41, 221-227.

Rall, W.F. and Fahy, G.M. (1985): Ice-free cryopreservation of mouse embryos at -196 °C by vitrification. Nature 313, 573-575.

Schmid, G., Lüdemann, H. D. and Jaenicke, R. (1975): High pressure effects on the activity of glycolytic enzymes. Biophys. Chem. 3, 90-98.

Schuster B. and Sleytr U.B. (2002). The effect of hydrostatic pressure on S-layer-supported lipid membranes. Biochim Biophys Acta 1563, 29-34.

Seki K. and Toyoshima M. (1998). Preserving tardigrades under pressure. Nature 395, 853-854.

Shaw J.M. and Kasai M. (2001). Embryo cryopreservation for transgenic mouse lines. Methods Mol Biol. 158, 397-419.

Shaw J.M., Diotallevi L. and Trounson A.O. (1991). A simple rapid 4.5 M dimethil-sulfoxide freezing technique for the cryopreservation and one-cell to blastocyst stage preimplantation mouse embryos. Reprod Fertil Dev. 3, 621-626.

Shaw J.M., Diotallevi L. and Trounson A.O. (1991). A simple rapid 4.5 M dimethil-sulfoxide freezing technique for the cryopreservation and one-cell to blastocyst stage preimplantation mouse embryos. Reprod Fertil Dev. 3, 621-626.

Shaw J.M. and Kasai M. (2001). Embryo cryopreservation for transgenic mouse lines. Methods Mol Biol. 158, 397-419.

Silva J.L., Foguel D. and Royer C.A. (2001). Pressure provides new insights into protein folding, dynamics and structure. Trends Biochem Sci 26, 612-618.

Spilimbergo S., Elvassore N. and Bertucco A. (2002). Microbial inactivation by high-pressure. The Journal of Supercritical Fluids 22, 55-63.

Takahashi, T., Kakita, A., Takahashi, Y., Yokoyama, K., Sakamoto, I. and Yamashina, S. (2001): Preservation of rat livers by supercooling under high pressure. Transplant. Proc. 33, 916-919.

Takahashi, T., Kakita, A.,Takahashi, Y., Sakamoto, I., Yokoyama, K., Fujiu, T., Yamashina, S., Tamaki, T., Takazawa Y. and Muratsubaki, R. (2000). Functional integrity of the rat liver after subzero preservation under high pressure. Transplant. Proc. 32, 1634-1636

Weber G. and Drickamer H.G. (1983). The effect of high pressure upon proteins and other biomolecules. Q Rev Biophys 16, 89-112.

Welch T.J., Farewell A., Neidhardt F.C. and Bartlett D.H. (1993). Stress response of Escherichia coli to elevated hydrostatic pressure. J Bacteriol 175, 7170-7177.

Wemekamp-Kamphuis H.H., Karatzas A.K., Wouters J.A. and Abee T. (2002). Enhanced levels of cold shock proteins in Listeria monocytogenes LO28 upon exposure to low temperature and high hydrostatic pressure. Appl Environ Microbiol 68, 456-63.

Wouters J.A., Jeynov B., Rombouts F.M., de Vos W.M., Kuipers O.P. and Abee T. (1999). Analysis of the role of 7 kDa cold-shock proteins of Lactobacillus lactis MG1363 in cryoprotection. Microbiology 145, 3185-3194.

Yager P. and Chang E.L. (1983). Destabilization of a lipid non-bilayer phase by high pressure. Biochim Biophys Acta 731, 491-494.

Yamanaka K., Fang L. and Inouye M. (1998). The CspA family in Escherichia coli: multiple gene duplication for stress adaptation. Mol Microbiol 27, 247-255.

IV. FINAL CONCLUSIONS

Recently, more attention is paid to the cryobiology of embryos being in later developmental stages. As the result of the developed embryo culturing conditions, in human in vitro fertilization (IVF) to lower the incidence of multiple gestations and improve implantation rates, the embryos can be cultured to the blastocyst stage, and transferred at the expanded, hatching or hatched stages (Khorram et al., 2000; Gardner et al., 1998; Yoon et al., 2001). In domestic animals, the extended culture of embryos gives a possibility to better pre-transfer evaluation; besides in some species (pig) later stages provide improved post thaw results. It was published (Ouhibi et al., 2000), that cryopreserved embryos undergo physicochemical changes that could inhibit hatching, thus contributing to unsuccessful conceiving, which also underlines the possible importance of transferring the embryos even later, at the hatched stage. Consequently, in the last years the interest is increasing in freezing of hatched blastocysts, though only a few papers have been published in this subject.

The quick freezing method used in our study combined dehydration with non-penetrating sucrose together with the penetrating EG or GLY. In the freezing experiments we used only those blastocysts that hatched early (140 -142 h after hCG injection) because viability of the delayed embryos is lower, and the freezing procedure might reduce it further.

As the outcome of the trial, ethylene glycol (EG) proved to be superior to glycerol (GLY). The best results were obtained with direct re-hydration of embryos cryopreserved in 3M EG (98% and 95%). However, these results did not show significant difference from the survival of the other groups cryopreserved in 3M EG. Results indicate, that 2 min equilibration provides enough time for EG to penetrate into the cells, also that the toxicity of EG in 3M concentration is negligible, since the 2 min and 30 min equilibration gave similar survival rates (95% and 94%). The results support the hypothesis that permeability increases as development proceeds. The significant drop in the survival rate when the concentration of EG was reduced to 1.5M (43% and 54%) indicated that the optimal concentration of EG for rapid freezing of hatching/hatched blastocysts is 3M.

Compared to the control group, all groups of embryos frozen with GLY showed a significantly reduced survival.

According to our results, the equilibration time did not affect the survival rates, indicating, that the toxicity of GLY might play a similar role in the poor survival rates in each of the groups. The best result with GLY was obtained with 20 min of equilibration, supports the principle that GLY, due to its higher molecular weight, penetrates slower into the cells.

At the in vivo experiments, we relied on the results of Zhu et al., (1996) and transferred the embryos to Day 3 pseudopregnant recipients, using 2 days asynchrony, in order to give the embryo more time to resume the normal developmental potential. Our results with EG were better than those obtained with GLY, though the differences were not significant. The pregnancy results obtained in our study were lower than those ones found in the literature, which can be explained with technical difficulties connected with housing of the animals and in-experiences of the person doing the transfer.

From the present study, it can be concluded, that zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium, using as short as 2 min equilibration time (that is conveniently enough to load the blastocysts into straws and seal them) at room temperature. The results also indicate that the mechanical protection of the zona pellucida is not vital during freezing in these stages.

Though our results with EG seem optimal, further experiments should be conducted using larger concentrations of non-permeating cryoprotectants applied either directly or step by step to dehydrate the embryos more before plunging. Pre-freeze equilibration of the embryos in the cryoprotectant solution at 0 °C instead of room temperature may also improve survival. At transfer, numerous embryos have been lost; main reason was that they attached to the wall of the glass pipette. Their shape also changed during moving them in between the drops (culture mediums), and in the straws and transfer pipettes. In my opinion, developing a standardised procedure for the transfer of zona-free blastocysts would improve in vivo success rates.

The cryopreservation of blastocyst-stage mouse embryos is well established. However we chose this developmental stage as a model to study the possible beneficial effects of high hydrostatic pressure (HHP) in the process of embryo-(cryo)preservation. Our investigations in this subject were based on the following two separate hypothesises:

1 HHP reduces substantially the freezing point of the pressure medium. Based on this phenomenon, we tried to build up a protocol, through which we would be able to keep and preserve embryos in their optimal culture medium, under pressure, unfrozen even around – 21 °C.

2 HHP induces the production of shock proteins in biological systems. Based on the alleged principle of cross-protection we tried to treat embryos with HHP prior to the procedure of freezing in order to improve their ability to resist the “shock” of freezing.

The phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level. In the first set of experiments, similarly, we intended to reduce the phase transition temperature of the embryo holding solution through the application of HHP with the aim to preserve embryos in their culture medium at temperatures below 0 °C, for a substantial time without freezing. We calculated that the freezing point of the embryo-holding medium at 200 MPa would be around minus 30°C – 35°C. We postulated that this range of temperature, without all the deleterious effects of the freezing process, would successfully preserve embryos provided, that they tolerate pressure of this magnitude well.

In order to find out if embryos survive at altered pressure conditions we studied the behaviour of embryos under pressure at room temperature. We found that embryos could survive a substantial amount of pressure without any visible change in their morphology (e.g. 90 MPa for 1 s or 30 MPa for 2 h). The maximum time the embryos survived under pressure reduced (>6 hours to 1 sec) with increasing pressure (from 10 MPa to 150 MPa). We also experienced a reversible morphological change after retrieving the embryos from the pressure chamber: embryos compacted depending on the magnitude and the duration of the applied pressure treatment. As for the next stage, we tried to improve the survival of the pressurized embryos, postulating that the pressure itself is not detrimental, but the sudden change of biophysical and biochemical features during the coming-up. We also intended to observe the effects of the two impacts, namely low temperature and high-pressure environment, jointly.

In the second series of trials, we tried to improve the survival rate of the pressurized embryos by gradual decompression. Our studies showed that the survival rate of the pressurized embryos increased strikingly if they were retrieved gradually. While 60 min at 90 MPa was lethal for all of the embryos, 80 % survived when 120 min gradual decompression was used. These findings convinced us that high pressure itself was not lethal. On the other hand, the survival of embryos pressurized at 0 °C reduced significantly. While at room temperature embryos had an average survival rate of 90% at 30 MPa for 45 min, none of the embryos survived the same impact at 0 °C. After 10 min or 5 min at 0 °C, none of the embryos survived at 60 MPa and at 90 MPa respectively, though at room temperature the survival rate was around 90 % in both cases.

The higher the pressure at a low temperature the less time the embryos survived; consequently we hypothesized that the metabolism of the embryos plays a more significant role in the adaptation to high hydrostatic pressure as the pressure rises.

Embryos pressurized at 0 °C were also decompressed gradually. Assuming that a slowed metabolism (due to low temperature) needs more time to restore the normal atmospheric equilibrium we also tried to lengthen the retrieval times, used successfully in the second experiment. The application of gradual decompression at low temperature did not have a beneficial effect on the embryo-survival.

Based on the present experiments the storage of embryos at subzero temperatures under high pressure is not applicable, since the two effects (pressure and low temperature) together significantly reduces survival even at 0°C.

In the second set of experiments, our aim was to explore the effect of a simple sublethal shock -hydrostatic pressure- to the survival capacity of the expanded mouse blastocysts to another “shock”: cryopreservation. Based on the results of previous publications conducted with microorganisms we postulated that HHP induces the production of shock proteins in the embryos, too. We also postulated, that similarly to the studies with bacteria, these changes would “cross-protect” the embryos at the “shock” of freezing.

Since pressure cannot be directly responsible for squeezing the water out of the blastocysts, we assumed, based on literature data that the compaction of the embryos was due to the consequences of pressure-induced production of different proteins (CSPs, HSPs), reversible alterations in protein structure and metabolic processes. For

this reasons only blastocysts, which compacted after pressurization, were used in the experiments. We applied a pressure impact of 60 MPa for 30 min – where approximately 90% of the embryos became compacted and survival was not different from the untreated control. According to the results the applied pressure treatment stunningly improved the in vitro development of the embryos after freezing. After 6 hours 98 % of the pressure treated blastocysts were morphologically (diameter, structural integrity, and general morphology) exactly identical to the control embryos and 95% of the blastocysts fully hatched within 20 hours, together with the controls. Embryos frozen without pressure treatment reexpanded only 20 hours after thawing. The proportion of the re-expanded blastocysts was significantly inferior to those receiving pressure treatment (only 29 % was at least 2/3 expanded). In addition, no embryos hatched from the non-treated group.
(Comment: we also cryopreserved embryos that were not compacted after the pressurization. The developmental speed, survival and hatching rates were reduced compared to the compacted ones. Due to the low number of embryos in this experiment, data is not presented.)

Our results showed that the applied pressure treatment prior to freezing could improve the in vitro developmental speed, survival and hatching rates of the embryos. Knowledge of this experiment can be useful in improving success rates in embryo-cryopreservation (and embryo-manipulation) of other mammalian species including the human. It can also be a model in improving the success rates with other type of cells, such as sperm, oocytes, stem cells, etc.

Further studies are required to support the deeper understanding of pressure assisted metabolic and structural changes of the pressurized embryos. The possible production of shock proteins and their role in cross-protection shall also be proved and studied, since our possibilities unfortunately did not include the tools of molecular biology. Similarly, more experiments are needed with different developemental stages of embryos, different cell types, different pressure/time parameters. Also, experiments should be conducted with expanded blastocysts which are non-compacted after the pressure treatment, because the compaction can also be a result of pressure-altered permeation and diffusion properties: the production of shock proteins may also be independent from this feature (in this case compaction can be a sign of the “sub-lethal zone”).

As an addition, there is even more to the use of HHP than this. By the application of pressure-shift freezing (sudden release of pressure, when the sample is cooled just close to the freezing point belonging to that magnitude of pressure) the ice nucleation rate can be greatly improved. As a result, numerous and fine ice nuclei are obtained, which grow into a massive number of small ice crystals, which are much less damaging. Also it would be interesting to observe the effects of other forms of ice in cryopreservation, for example ice-III., that has a higher density than water and also a finer texture. Nevertheless, this ice form is only stable under pressures (around 250 MPa) that limit its practical application.

V. RESEARCH ACHIEVEMENTS

1 Zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium. As short as 2 min equilibration time provides sufficient time for the ethylene glycol to penetrate the cells because of the increased permeability of the hatched blastocyst stage.

2 Introduction of high hydrostatic pressure in the process of cryopreservation.

3 Embryos can survive a substantial amount of pressure without any visible change in their morphology. The maximum time the embryos survive under pressure reduces with increasing pressure. Transferring the survived pressurized embryos, they develop into healthy pups as well as controls.

4 Embryos go through a reversible morphological change after the retrieval from the pressure chamber: they become compacted depending on the magnitude and the duration of the applied pressure treatment.

5 The survival rate of the pressurized embryos increases strikingly if they are retrieved gradually.

6 The survival of embryos pressurized at 0 °C reduces significantly. The higher is the pressure at a low temperature the less time the embryos survive.

7 We showed that pressure treatment prior to freezing stunningly improves in vitro the speed of reepandation, survival and hatching rates of the embryos.

VI. BRIEF SUMMARY IN HUNGARIAN

Az első sikeres embriófagyasztásról szóló közlemény több mint 30 évvel ezelőtt, Whittingham (1972) tollából jelent meg. A kriobiológusok érdeklődése elsősorban a még zona pellucidával rendelkező (ki nem bújt) embriók fagyasztására irányult. Ennek hátterében az áll, hogy a gyakorlatban a haszonállatok embrió-átültetésénél a zona pellucida (ZP) megléte fontos tényező például a fertőző betegségek átvitelének csökkentése szempontjából, továbbá az embriótenyésztés feltételeit a korai stádiumú, ZP-val rendelkező embriókra kísérletezték ki a legnagyobb alapossággal. Korábban az embriókat csak rossz hatásfokkal lehetett az expandált illetve kibújt blasztociszta stádiumig tenyészteni, ennek ellenére az első fagyasztott embrióból származó borjú, bárány és malac ilyen stádiumú fagyasztott embriók sikeres átültetéséből született.

Az utóbbi években, a tenyésztési körülmények lényeges javulásával mind nagyobb hangsúly helyeződik a későbbi fejlődési szakaszban lévő, illetve a ZP-val nem rendelkező embriók fagyaszthatóságával kapcsolatos vizsgálatokra. A humán gyakorlatban egyre nagyobb teret nyer a blasztociszta embriók beültetése, amitől a szakemberek - a beültetett embriók kisebb száma miatt- az ikerterhességek ritkább előfordulását várják. Ugyanakkor a fagyasztott, ZP-val rendelkező embriók beültetésével kapcsolatban Ouhibi (2000) azt írja, hogy a fagyasztás során olyan fiziko-kémiai folyamatok mennek végbe, amelyek gátolhatják a blasztociszta kibújását, ezáltal akadályozva az embrió megtapadását. Humán embriók fagyasztására általában propilénglikolt (PG) és szaharózt tartalmazó oldatot használnak. Shaw (1995) PG és etilénglikol (EG) alkalmazásával sikeresen fagyasztott expandált és kibújt egér blasztocisztákat hagyományos módszerrel. Vizsgálataiban azonban egy csoportban kezelte az említett fejlődési stádiumokat, külön kísérletet nem végzett ZP nélküli embriókkal. Egy korábbi (1991) kísérlet-sorozatban 4,5 M-os DMSO-t használva Shaw egyszerű gyors-fagyasztásos módszert alkalmazott különböző fejlődési szakaszban lévő embriókon. Azt tapasztalta, hogy az embrió kora illetve az ekvilibráltatás hőmérséklete és ideje befolyásolja az embriók felengedés utáni túlélését: 0 C°-on 3 illetve 6 perces ekvilibráltatással 40%-os, 10 és 60 perces ekvilibráltatással 40-60%-os túlélést ért el kibújt blasztocisztákon, míg szobahőmérsékleten 3 percig ekvilibrálva az embriókat csak 20 %-uk fejlődött a felmelegítés után.

Zhu és munkatársai 1996-ban kibújt egér blasztocisztákat ekvilibráltak egy vagy két lépésben különböző ideig, majd vitrifikálták őket kétféle, etilén-glikolt (EG), vagy

glicerint (GLY), valamint ficollt és szaharózt tartalmazó oldatokban. Etilén-glikollal a legmagasabb túlélési arányt (65%) az egylépéses ekvilibráltatással érték el, olyan embriókkal, amelyek a leghamarabb bújtak ki a ZP-ból. Glicerint használva jobbak voltak az eredmények (89-94%) a kétlépéses ekvilibráltatáskor. A legmagasabb in vivo túlélés 44% volt, 3, illetve 4 napos álvemhes recipienseket használva.
A jelen dolgozatban közölt kísérletek célja az EG-al, illetve GLY-nel történő gyorsfagyasztásnak a kibújt egér blasztociszták in vitro és in vivo túlélésére gyakorolt hatásának a tanulmányozása volt. A fagyasztás előtti különböző ekvilibráltatási idők és a fagyasztás utáni rehidrációs idők hatását is vizsgáltuk.

A gyors fagyasztás egyszerű és hatékony módja az embriófagyasztásnak. Gyors fagyasztáskor a felhasznált krioprotektív anyag töménysége a fagyasztó oldatban magasabb a hagyományos fagyasztásban használtnál, viszont alacsonyabb a vitrifikáció során alkalmazottnál, ezáltal a toxicitása is csekélyebb. A leírt kísérletben használt gyors fagyasztási módszer összefűzte a nem penetrálódó fagyálló vegyület – szaharóz - dehidráló hatását a penetrálódó EG vagy GLY hatásával.
Vizsgálatainkban csak a ZP-ból elsőként kibújt blasztocisztákat (140-142 órával a hCG beadása után) használtunk, mivel a lassúbb fejlődésű embriók életképessége csekélyebb, és ez még tovább romlik a fagyasztási folyamat során.
A kísérletünkben használt két krioprotektív anyagnak az embrió-túlélésre gyakorolt hatását összehasonlítva az EG lényegesen jobbnak bizonyult a GLY-nál. A legmagasabb túlélést (98% és 95%) közvetlen rehidrálással értük el 3 mólos EG-t használva, noha ez az eredmény nem mutatott szignifikáns különbséget a többi 3 M EG csoporthoz hasonlítva.
Az eredmények azt mutatták, hogy az EG számára két perc is elegendő idő a sejtekbe való penetrálódásra, valamint azt is, hogy a 3 mólos töménység toxicitása elhanyagolható, mivel a 2 perces és a 30 perces ekvilibráltatás hasonló túlélési eredményeket hozott (95% és 94%).
Vizsgálataink adatai támogatják azt a feltevést, miszerint az embrióknak a különböző anyagokkal szemben mutatott membrán-permeábilitási tulajdonsága a fejlődésük előrehaladtával változik, általában növekszik: EG gyorsabban bejut a kibújt blasztocisztákba, mint a ZP-vel rendelkezőkbe.
Zhu és munkatársai (1996) egyik kísérletében a 40%-os EG-ban, szobahőmérsékleten végzett 5 perces ekvilibráltatás 6 % -ra csökkentette a kibújt blasztociszták túlélését.

Összevetve a saját és az idézett vizsgálatot valószínűsíthetjük, hogy a rendkívül magas koncentrációjú, gyorsan penetrálódó EG toxikus hatása lényegesen kifejezettebb, mint a kevésbé tömény 3M-os oldaté.

A túlélési arány jelentős csökkenése (43 illetve 54 %-ra) az EG töménységének 3M-ról 1.5 M-ra csökkentésekor azt sugallja, hogy a kibújt blasztociszták fagyasztásához kísérletünkben az optimális töménység a 3M.

A glicerollal fagyasztott embriók túlélése lényegesen rosszabb volt a kontroll csoportéhoz képest. Ezen eredményeink ellentétesek Zhu és munkatársai (1996) által tapasztaltakkal, akik úgy találták, hogy glicerinnel jobb túlélési eredmények érhetők el, mint etilén glikollal; noha az általuk használt krioprotektív anyag koncentrációja lényegesen nagyobb volt, mint a mi kísérletünkben. Abból, hogy eredményeink szerint az ekvilibráltatási időnek nem volt befolyása a túlélésre, arra következtethetünk, hogy a GLY toxicitása lehet a legfőbb oka az embriók szerény túlélési arányának ezekben a kísérleti csoportokban. Zhu vizsgálatainak a tükrében az eredményeink azt mutatják, hogy az általunk használt 3 mólos töménység nem optimális kibújt blasztociszták glicerinnel történő gyors fagyasztására. A glicerin, a nagyobb molekulasúlya következtében, lassabban jut be a sejtekbe; ezt támasztotta alá az a megfigyelés, miszerint a csoportok közül a 20 perces ekvilibráltatási idő adta a (nem szignifikáns) legjobb eredményt.

Shaw és munkatársai (1991) DMSO-val végzett kísérleteikben 0 C°-os ekvilibráltatás mellett tapasztaltak a mienkéhez hasonló eredményeket, viszont szobahőmérsékleten történt ekvilibráltatáskor esetükben a túlélés számottevően csökkent.

Az in vivo értékeléskor a kibújt blasztocisztákat, 2 napos asszinkronitást használva 3 napos álvemhes recipiensekbe ültettük be. Kísérletünkben az EG-al elért in vivo túlélési arány, noha nem szignifikánsan, de jobb volt a glicerinnel elért eredményeknél. Zhu és munkatársai nagyobb túlélési arányról számoltak be kibújt blasztocisztákkal 1 illetve 2 napos aszinkronitással történő beültetéseket követően, ami azt jelentheti, hogy a vitrifikáció kevesebb károsodással jár, mint a gyors fagyasztás (az irodalomhoz képest szerényebb in vivo eredményeinkhez a beültetéssel és állattartással járó technikai nehézségek is hozzájárultak).

Kísérleteink alapján kijelenthetjük, hogy a gyors fagyasztási eljárás alkalmas a kibújt egér blasztociszták fagyasztására; ezen belül is jó eredmények érhetők el 3 M töménységű EG-t tartalmazó fagyasztó oldattal, mindössze 2 perces ekvilibráltatási idővel (mely elegendő az embriók műszalmába töltésére), akár szobahőmérsékleten.

Eredményeink szerint ebben a fejlődési szakaszban már nem számottevő fagyasztáskor a zóna pellucida mechanikai szerepe az embrió védelmében.

Egér-blasztociszták sikeres fagyaszthatóságáról számos cikk beszámol. A második kísérletsorozatunkkal tehát nem egy jól működő rendszert szerettünk volna még hatékonyabbá tenni, hanem egy új, eddig ilyen formában embriófagyasztással kapcsolatban még nem közölt változó, a hidrosztatikus nyomás (HHP) lehetséges hasznosítását vizsgáltuk, két, egymástól alapvetően különböző alapon:

1, HHP hatására a folyadékok fagyáspontja csökken: 210 MPa nyomás alatt a tiszta víz fagyáspontja -21C°. A folyadékok e fizikai tulajdonságát felhasználva célunk az volt, hogy nyomás alatt, a tápfolyadék fagyáspontját számottevően lecsökkentve, hűtve kíséreljük meg az embriók jó hatásfokú, tartós tárolását. Ezáltal az embriókat optimális közegben, a fagyással járó összes károsodástól mentesen lehetne tárolni, feltéve, hogy a jelentősen megemelkedett hidrosztatikus nyomást károsodás nélkül képesek elviselni.

2, HHP hatására biológiai rendszerekben sokk-fehérjék termelődhetnek, melyek más külső behatásokkal szemben is javíthatják az adott rendszer túlélését. Élelmiszer-mikrobiológia témakörben számos dolgozat jelent meg e témában. A kísérletek középpontjában az a megfigyelés volt, hogy szubletális nyomás-sokk és hő-sokk együttes alkalmazása nemhogy csökkentette egy adott élelmiszer patogén csira tartalmát, hanem épp ellenkezőleg, javította a baktériumok túlélését. A hidrosztatikus nyomás e biológiai hatását kihasználva azt vizsgáltuk, hogy egy, a fagyasztási eljárás előtt alkalmazott nyomás-impulzus javítja-e az embriók fagyasztás utáni túlélését, az említett feltételezett „kereszt-védelem” alapján.

Kísérleteink soron következő lépéseit mindig az előző vizsgálatok eredményei alapján terveztük, mivel korábbi közleményekre támaszkodni nem tudtunk.

Első lépésként azt kellett megvizsgálnunk, hogy az egér embriók (blasztociszták) túlélését, továbbfejlődését miképp befolyásolja a megnövekedett hidrosztatikus nyomás. A blasztocisztákat tápfolyadékban buborék nélkül műszalmába szívva nyomáskamrába helyeztük, ahol a nyomást közvetítő közeg víz volt. Ezt követően, kb. 10 MPa/perc sebességgel 10-150 MPa nagyságú hidrosztatikus nyomást generáltunk a nyomáskamrában, melyet 1 s – 3 óráig fenntartottunk (szobahőmérsékleten), majd kb. 2

másodperces dekompressziót követően az embriókat normál légköri nyomásra hozva tápfolyadékban, Petri csészében termosztátba helyeztük és továbbfejlődésüket egészen a kibújt stádiumig figyelemmel kísértük.

A kísérlet során a következőket figyeltük meg:

1. Az embriók túlélését az alkalmazott hidrosztatikus nyomás illetve a nyomás alatt eltöltött idő jelentősen befolyásolja. Az embriók túlélése és továbbfejlődésük aránya nem különbözött szignifikánsan a kezeletlen kontrollhoz viszonyítva az 150 MPa/1 s, 90 MPa/30 min, 60 MPa/120 min illetve a 10 MPa/180 min beavatkozásoknál kevesebb ideig tartó vagy kisebb nyomású behatásoknál. Ezen értékeket meghaladó behatás az embriók túlélését szignifikánsan rontotta (pl. 150 MPa/15 min –nél 0% túlélés).
2. A blasztociszták egy bizonyos nyomás/idő értékig (pl. 60 MPa/10 min) morfológiai változás nélkül jöttek elő; ezt meghaladó kezelésnél viszont (pl. 60 MPa/30 min) morfológiai változáson mentek át: a blasztocöl eltűnt, a blasztociszta „kompaktálódott”. Ebből az állapotból, az 1. pontban leirt értékek alatt, 3-4 órás in vitro tenyésztéssel visszaalakultak, és a kontroll embriókkal azonos időben és arányban kibújtak.
3. Az 1. pontban leirt értékekkel kezelt embriók (kompaktálódott és morfológiájában változatlan egyaránt) beültetve a kontroll embriókhoz hasonló arányban tapadtak meg, míg a morfológiailag „nem túlélt”-nek ítélt embriók közül megtapadás nem volt az in vivo kísérletekben.

Az első kísérletsorozatból megtudtuk, hogy az embriók képesek nagy nyomást is túlélni, viszont a számunkra megcélzott nyomástartományban (> 100MPa) a 90 % körüli túlélés csak nagyon rövid ideig tartó behatással tartható. Így a következő sorozatban azt próbáltuk kideríteni, hogy fokozatos dekompresszióval javítható-e az embriók túlélése.

A fent említett körülményeket annyiban változtattuk meg, hogy 2 másodperces dekompresszió helyett 30-180 percet alkalmaztunk. Az eredmények alapján kijelenthettük, hogy a fokozatos felhozatal jelentősen növelte az embriók túlélését. Míg 90 MPa/30 min kb. 50%-os túlélést eredményezett 2 s dekompresszióval, addig 90 és 120 perces dekompressziót követően 98 % túlélést sikerült elérnünk.

A harmadik sorozatban a két „sokk”, a nyomás és az alacsony hőmérséklet együttes hatását vizsgáltuk. Több kísérletet végeztünk 90 MPa, 60 MPa illetve 30 MPa nyomást használva -8, -5 és -2 C°-on, de egyetlen embrió sem élte túl a beavatkozást. 0 C°-on

végzett vizsgálatok azt mutatták, hogy 30, 60 és 90 MPa nyomás hatására at embriók 30, 10 illetve 5 perc után elpusztulnak, noha szobahőmérsékleten a túlélésük nem befolyásolt. A második kísérletben használt szakaszos visszahozatalnak túlélést javító hatása nem volt.

A tapasztalatok alapján megállapíthatjuk, hogy az adott gondolatmenet alapján az embriók tartós tárolása ily módon nem ésszerű, hiszen a nyomás illetve az alacsony hőmérséklet együtt túl nagy sokknak bizonyult. A negatív eredmények ellenére a kísérlet tanulságai felhasználhatóak egyéb biológiai anyag fagyasztási kísérleteihez.

A sokk-fehérjék termelődésének indukálásán alapuló kísérletekben az első sorozatra alapozva kiválasztottunk egy paraméter-párt, melynél a morfológiai átalakulása a blasztocisztáknak már 90-100 %-ban megtörtént, viszont az in vitro (és in vivo) túlélés és továbbfejlődés a kontroll embriókétól nem különbözik (90-100%). Arra a feltételezésre alapoztuk a választást, hogy mivel a hidrosztatikus nyomás a folyadékokban minden ponton, minden irányból hat, ezért nem lehet oka a blasztociszták morfológiai átalakulásának: tehát a kompaktálódást a fehérjék nyomás hatására történő reverzibilis átalakulásának illetve fehérjék (sokk-fehérjék) termelődésének következményeként értékeltük.

Hatvan MPa nyomást alkalmaztunk szobahőn, 30 percig. A két másodperces dekompressziót követően a kompaktálódott blasztocisztákat Nowshari és Brem által 1998-ban közölt módszer szerint fagyasztottuk (7 M etilén glikol, 0,5 M szaharóz), majd a felengedés után termosztátban tenyésztettük a kibújt stádiumig.

Az eredmények azt mutatták, hogy a nyomással előkezelt embriók a felengedést követő 6 órával már 98 %-ban re-expandálódtak, 20 órával pedig 95 % kibújt, míg a nyomás előkezelés nélkül fagyasztott embrióknak 6%-a a felengedést követő 6. órában volt még csak ½ expandált, 20 óra elteltével 29 % volt legalább 2/3 expandált és 0 % kibújt.

Nyomás előkezeléssel tehát az embriók visszaalakulásának sebessége gyorsult, a túlélés illetve a kibújás aránya jelentősen javult. Mivel eredményeink illetve a feltételezett biológiai háttér nincsenek molekulár-biológiai módszerekkel alátámasztva, indokolt lenne a nyomás hatására bekövetkezett biológiai, biokémiai változások behatóbb vizsgálata (nyomás hatására bekövetkező anyagcsere változásainak vizsgálata, fehérjék tulajdonságainak vizsgálata..); illetve a módszer szélesebb körű (nagyobb esetszám, más fejlődési stádium, más faj, más biológiai anyag) kipróbálása.

1 Kibujt egér-blasztociszták jó hatásfokkal fagyaszthatók egyszerű gyors-fagyasztásos módszerrel, 3 mólos töménységű etilén glikol alkalmazásával. Kettő perces ekvilibrálás elegendő időt biztosít a krioprotektánsnak a sejtekbe történő penetrálódásra.

2 Hidrosztatikus nyomás beillesztése a krioprezerváció folyamatába.

3 Expandált blasztociszták minden látható morfológiai változás nélkül igen nagy hidrosztatikus nyomást képesek elviselni. A nyomásérték illetve a nyomás alatt eltöltött idő növekedésével az embriók túlélési aránya csökken. Nyomással kezelt embriók, melyek in vitro továbbfejlődtek, beültetve a kezeletlen kontrollokhoz hasonló arányban születtek meg.

4 Az alkalmazott nyomás nagyságától és idejétől függően az embriók reverzibilis morfológiai változáson mennek át: kollabálnak.

5 A nyomással kezelt embriók túlélése szakaszos dekompresszióval szignifikánsan javítható.

6 Ha a nyomáskezelés alacsony hőmérsékleten (0 °C –on) zajlik, az embriók túlélése szignifikánsan rosszabb.

7 A fagyasztási protokollt megelőző nyomás-impulzus szignifikánsan növeli az embriók felengedést követő túlélését, in vitro továbbfejlődését (reexpandálódás sebessége és hatching).

VII. LIST OF PUBLICATIONS

CS. PRIBENSZKY, S. CSEH, L. SOLTI: IN VITRO SURVIVAL OF EXPANDED, HATCHING OR HATCHED BLASTOCYSTS FROZEN RAPIDLY IN ETHYLENE GLYCOL AND RE-HYDRATED IN SUCROSE OR DPBS. PROCEEDINGS OF THE 5TH ANNUAL CONFERENCE OF THE EUROPEAN SOCIETY FOR DOMESTIC ANIMAL REPRODUCTION, 2001, AUSTRIA, VIENNA (Abstract)

CS. PRIBENSZKY, S. CSEH, L. SOLTI.: IN VITRO SURVIVAL OF HATCHING AND HATCHED MOUSE BLASTOCYSTS CRYOPRESERVED BY RAPID FREEZING. THERIOGENOLOGY, 2002. VOL. 57. NO.1. P. 476. (Abstract)

M. MOLNÁR, CS. PRIBENSZKY, S. CSEH, L. SOLTI: INVESTIGATION ON VIABILITY OF EMBRYOS AFTER EXPOSING TO HIGH HYDROSTATIC PRESSURE. THERIOGENOLOGY, 2002. VOL. 57. NO.1. P. 506. (Abstract)

CS. PRIBENSZKY, S. CSEH, L. SOLTI: IN VITRO SURVIVAL OF EXPANDED MOUSE BLASTOCYSTS PRESSURIZED AT ROOM TEMPERATURE AND AT 0 C°. REPRODUCTION IN DOMESTIC ANIMALS, 2002. VOL. 37., NO. 4. P. 247. (Abstract)

CS. PRIBENSZKY, M. MOLNÁR, S. CSEH, L. SOLTI: VIABILITY OF EMBRYOS AFTER EXPOSING TO HIGH HYDROSTATIC PRESSURE. THERIOGENOLOGY 2003. JAN. VOL.59. NO.1. P. 329. (Abstract)

CS. PRIBENSZKY, S. CSEH, L. SOLTI: RAPID FREEZING OF HATCHED ZONA-FREE MOUSE BLASTOCYSTS. THERIOGENOLOGY 2003. JAN. VOL.59. NO.1. P. 309. (Abstract)

CS. PRIBENSZKY, S. CSEH, ZS. ABONYI-TÓTH AND L. SOLTI: SURVIVAL OF RAPIDLY FROZEN HATCHED MOUSE BLASTOCYSTS. ZYGOTE 2003. 11. PP. 361-366.

C. PRIBENSZKY , M. MOLNAR , S. CSEH AND L. SOLTI: EFFECTS OF PREVIOUS PRESSURE TREATMENT ON THE SURVIVAL AND DEVELOPMENTAL SPEED OF EXPANDED MOUSE BLASTOCYSTS FROZEN RAPIDLY (PILOT STUDY).

REPRODUCTION, FERTILITY AND DEVELOPMENT 2004. VOL. 16 . NO. 1 & 2 P. 181. (Abstract)

CS. PRIBENSZKY, M. MOLNÁR, S. CSEH, L. SOLTI: SURVIVAL OF MOUSE BLASTOCYSTS AFTER LOW TEMPERATURE PRESERVATION UNDER HIGH PRESSURE. ACTA VETERINARIA HUNGARICA 2004. (ACCEPTED FOR PUBLICATION)

PRIBENSZKY CSABA, CSEH SÁNDOR, ABONYI-TÓTH ZSOLT, SOLTI LÁSZLÓ: KIBÚJT EGÉR-BLASZTOCISZTÁK GYORS FAGYASZTÁSA - MÁSODKÖZLÉS. MAGYAR ÁLLATORVOSOK LAPJA 2004. (ACCEPTED FOR PUBLICATION)

CS. PRIBENSZKY, M. MOLNÁR, S. CSEH, L. SOLTI: IMPROVING POST-THAW SURVIVAL OF CRYOPRESERVED MOUSE BLASTOCYSTS BY HYDROSTATIC PRESSURE CHALLENGE. 2004. ANIMAL REPROUCTION SCIENCE (ACCEPTED FOR PUBLICATION)

VIII. ACKNOWLEDGEMENTS

I would like to thank my tutor, Sándor Cseh, that he introduced me into the science of embryology, showed me the example of being precise, critical, thorough and deliberate. I am thankful to our dean and head of department, László Solti, who really gave me a lot of enthusiasm to my work. I am also grateful to Sándor Cseh and László Solti for their useful advices and their time and effort in reviewing my papers.

I am grateful that I had the chance to work with my friend, Miklós Molnár from the Technical University, without whom it would be very unlikely that I get involved in the experiments with hydrostatic pressure. I really enjoyed his support and our inspiring brain storming sessions.

Finally yet importantly, I would like to say thanks to my parents, friends, girlfriends and colleagues who tolerated me in the hard days of thesis- and article-writing, and at the times when I spent a lot of days, nights, weeks and weekends in the lab being deeply involved in the “pressure project”.

IX. LIST OF REFERENCES

Abe, F. and Horikoshi, K. (1995). Hydrostatic pressure promotes the acidification of vacuoles in Saccharomyces cerevisiae. FEMS Microbiol Lett 130. 307-312.

Abe, F. and Horikoshi, K. (1997). Vacuolar acidification in Saccharomyces cerevisiae induced by elevated hydrostatic pressure is transient and is mediated by vacuolar H+-ATPase. Extremophiles 1. 89-93.

Abe, F. and Horikoshi, K. (1998). Analysis of intracellular pH in the yeast Saccharomyces cerevisiae under elevated hydrostatic pressure: a study in baro- (piezo-) physiology. Extremophiles 2. 223-228.

Abe, F., Kato, C. and Horikoshi, K. (1999). Pressure-regulated metabolism in microorganisms. Trends Microbiol 7. 447-453.

Aldridge, B. E. and Bruner, L. J. (1985). Pressure effects on mechanisms of charge transport across bilayer membranes. Biochim Biophys Acta 817. 343-354.

Arav, A. and Zeron, Y. (1997). Vitrification of bovine oocytes using modified minimum drop size technique MDS is effected by the composition and concentration of the vitrification solution and by the cooling conditions. Theriogenology 47. 341.

Bagis, H., Odaman, H., Sagirkaya, H. and Dinnyes, A. (2002). Production of transgenic mice from vitrified pronuclear-stage embryos. Mol Reprod Dev. 61. 173-9.

Bagis, H., Sagirkaya, H., Mercan, H.O. and Dinnyes, A. (2004). Vitrification of pronuclear-stage mouse embryos on solid surface (SSV) versus in cryotube: comparison of the effect of equilibration time and different sugars in the vitrification solution. Mol Reprod Dev. 67. 186-92.

Baguisi, A., Arav, A., Crosby, T.F., Roche, J.F. and Boland, M.P. (1987). Hypothermic storage of sheep embryos with antifreeze proteins: development in vitro and in vivo. Theriogenology 48. 1017-1024.

Balmaceda, J.P., Heitman, T.O., Garcia, M.R., Pauerstein, C.J. and Pool, T.B. (1986). Embryo cryopreservation in cynomolgus monkeys. Fertil. Steril. 45. 403-406.

Bank, H. and Mauer, R.R. (1974). Survival of frozen rabbit embryos. Exp. Cell Res. 89. 188.

Bernard, A. and Fuller, B.J. (1996). Cryopreservation of human oocytes: A review of current problems and perspectives. Hum Reprod Update 2. 193-207.

Biery, K.A., Thomas, W.K. and Kraemer, D.C. (1986). Effect of cryoprotectant on mouse embryo development following rapid freezing. Theriogenology 31. 223.

Bilton, R.J. (1980). Preservation of embryos of the large domestic species. Proc. 9th Int. Congr. Anim. Reprod. And A.I. 2. 245.

Bilton, R.J. and Moore, N.W. (1976). In vitro culture, storage and transfer of goat embryos. Aust. J. Biol. Sci. 29. 125.

Bilton, R.J. and Moore, N.W. (1979). Factors affecting the viability of frozen stored cattle embryos. Aust. J. Biol. Sci. 32. 11.

Borland, R.M., Biggers, J.D. and Lechene, C.P. (1976). Kinetic aspects of rabbit blastocoel fluid accumulation: an application of electrone probe analysis. Dev. Biol. 50. 201.

Bracke, C. and Niemann, H. (1995). New aspects in the freezing of embryos from livestock. In: Proc.11th Scientific Meeting of European Embryo Transfer Association. Fondation Marcel Merieux, Lyon, France. 101-111.

Bridgeman, P.E. (1970). The physics of high pressure. New York: Dover

Bridgman, P.W. (1911). Water in the liquid and five solid forms under pressure. Proceedings of the American Academy of Arts and Science 47. 441–558.

Bui-Xuan-Nguyen, N., Heyman, Y. and Renard, J.P. (1984). Direct freezing of cattle embryos after partial dehydration at room temperature. Theriogenology 22. 389-399.

Butz, P. and Ludwig, H. (1986). Pressure inactivation of microorganisms at moderate temperatures. Physica B+C 139-140. 875-877.

Castellini, M. A., Rivera, P. M. and Rivera, J. M. (2002). Biochemical aspects of pressure tolerance in marine mammals. Comp Biochem Physiol A Mol Integr Physiol 133. 893-899.

Chen, S.U., Lien, Y.R., Chen, H.F., Chao, K.H., Ho, H.N. and Yang, Y.S. (2000). Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Human Reproduction, 15. 2598-2603.

Chupin, D. (1986). Quick freezing of bovine blastocysts. Theriogenology 25. 147.

Chupin, D. (1987). Quick freezing of day 7 bovine blastocysts: Optimum parameters of dehydration step. Theriogenology 27. 219.

Cseh, S. (1983). Szarvasmarha-embriók mélyhűtése. Magy. Áo. Lapja, 38. 339.

Cseh, S., Horlacher, W., Brem, G., Corselli, J., Seregi, J., Solti, L. and Bailey, L. (1999). Vitrification of mouse embryos in two cryoprotectant solutions. Theriogenology 52. 103-113.

Cseh, S., Corselli, J., Nehlsen-Cannarella, S.L., Bailey, L.L. and Szalay, A.A. (1997). The effect of quick-freezing in ethylene glycol on morphological survival and in vitro development of mouse embryos frozen at different praeimplantation stages. Theriogenology 48. 43-50.

Cseh, S., Mahboubi, Zine El Abidine, Bilton, R.J. and Moore, N.W. (1984). Mélyhűtött juhembriók sikeres átültetése. Magy. Áo. Lapja, 39. 72.

Csermely, P., Schnaider, T., Sőti, Cs., Prohászka, Z. and Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol. Ther. 79. 129-168.

Derzelle, S.,Bernard, H.,Thierry, F.,Jean, D. and Pascal, H. (2003). Improved adaptation to cold-shock, stationary-phase, and freezing stresses in Lactobacillus plantarum overproducing cold-shock proteins. Applied And Environmental Microbiology 69. 4285-4290.

DeVries, A.L. (1984). Role of glycopeptides and peptides in inhibition of crystallization of water in polar fishes. Trans. R. Soc. Lond. B304. 575-588.

Dinnyes, A., Dai, Y., Jiang, S. and Yang, X. (2000). High developmenal rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol. Reprod. 63. 513-518.

Dobrinsky, J.R. and Johnson, L.A. (1994). Cryopreservation of porcine embryos by vitrification: a study of in vitro development. Theriogenology 42. 25-35.

Dobrinsky, J.R., (1996). Cellular approach to cryopreservation of embryos. Theriogenology 45. 17–26.

Dobrinsky, J.R. (2002). Advancements in cryopreservation of domestic animal embryos. Theriogenology 57. 285-302.

Dresser, B.L., Gelwicks, E.J., Wachs, K.B. and Keller, G.L. (1988). First successful transfer of cryopreserved feline (felis catus) embryos resulting in live offspring. J. Exp. Zool. 246. 180-186.

Dumoulin, J.C., Bergers-Janssen, J.M., Pieters, M.H., Enginsu, M.E., Geraedts, J.P. and Evers, J.L. (1994). The protective effects of polymers in the cryopreservation of human and mouse zonae pellucidae and embryos. Fertil Steril 62. 793-798.

Elford, B.C. and Walter, C.A. (1972). Preservation of structure and function of smooth muscle cooled to -79 degrees C in unfrozen aqueous media. Nature: New Biology, Volume 236. 58-60.

Ernest, C., Weller, K. P. and Weller, K. P. (1966). The effect of hydrostatic pressure on the synthetic processes in bacteria. Biochim Biophys Acta 112. 573-580.

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Baley, H. and Toner, M. (2000). Intracellular trehalose improves the survival of cryopreserved mammalian cells. Nature Biotech. 18. 163-167.

Fahing, M.L. and Garcia, M.A. (1992). Status of cryopreservation of of embryos from domestic animals. Cryobiology 29. 1-18.

Fahy, G.M. (1980). Analysis of "solution effects" injury: rabbit renal cortex frozen in the presence of dimethyl sulfoxide. Cryobiology, 17. 371-388.

Fahy, G.M. (1986). The relevance of cryoprotectant `toxicity` to cryobiology. Cryobiology 23. 1-13.

Fahy, G.M., MacFarane, D.R., Angell, C.A. and Meryman, H.T. (1984). Vitrification as an approach to cryopreservation. Cryobiology 21. 407–426.

Franks, F. (1982). The properties of aqueous solutions at subzero temperatures. In: Water: a comprehensive treatise. Vol. 7. (Ed.: Franks, F.) Plenum Press, New York

Gardner, D.K., Vella, P., Lane, M., Wagley, L., Schlenker, T. and Schoolcraft, W.B. (1998). Culture and transfer of humane blastocysts increases implantation rates and reduces the need for multiple embryo transfers. Fertility and Sterility 69. 84-88.

Gomez, M.C., Pope, E., Harris, R., Mikota, S. and Dresser, B.L. (2003). Development of in vitro matured, in vitro fertilized domestic cat embryos following cryopreservation, culture and transfer. Theriogenology 60. 239–251.

Gordon-Kamm, W.J. and Steponkus, P.L. (1984). Lamellar-to-hexagonal II phase transitions in the plasma membranes of isolated protoplasts after freeze-induced dehydration. Proc. Natl. Acad. Sci.USA 81. 6373-6378.

Graumann, P.L. and Marahiel, M.A. (1999). Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in Bacillus subtilis. Arch Microbiol 171. 135-138.

Gross, M. and Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. Eur J Biochem 221. 617-630.

Gutierrez, A., Artiga, C., Garde, J. and Pintado, B. (1993). Effect of equilibration period and cryoprotectant in quick freezing of mouse morulae. Theriogenology 39. 229.

Hafez, E.S.E. (1993). Preservation and cryopreservation of Gametes and Embryos. Lea and Febinger, Philadelphia. 503-525.

Hahn, J., Hahn, R., Becze, J., Mészáros, J., Perjés, I. and Szilvássy, B. (1978). Megszületett az embrióátültetésből származó első borjú Magyarországon. Magy. Áo. Lapja, 33. 200.

Han, Y.M., Kim, S.H., Park, J.S., Park, I.Y., Kang, Y.K., Lee, C.S., Koo, D.B., Lee, T.H., Yu, D.Y., Kim, Y.H., Lee, K.J. and Lee, K.K. (2000). Blastocyst viability and generation of transgenic cattle following freezing of in vitro produced, DNA-injected embryos. Anim. Reprod. Sci. 63. 53-63.

Haraszti, J. and Rónai, G. (1977). Petesejt-transzplantációs vizsgálatok nyulakban és juhokban /előzetes közlemény/. Magy. Áo. Lapja, 32. 429.

Heape, W. (1881). Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. Proc. R. Soc. London, 48. 457.

Heyman, Y., Vincent, C., Garnier, V. and Cognie, Y. (1987). Transfer of frozen-thawed embryos in sheep. Vet. Rec. 120. 83-85.

Hochi, S., Terao, T., Kamei, M., Kato, M., Hirabayashi, M. and, Hirao M. (2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure Theriogenology. 15. 267-75.

Hochi, S., Semple, E. and Leibo, S.P. (1996). Effect of cooling and warming rates during cryopreservation on survival of in vitro-produced bovine embryos. Theriogenology 46. 849-858.

Horlacher, W. and Brem, G. (1994). Comparison of 3 different vitrification methods for cryopreserving mouse embryos. Theriogenology 41. 218,1994.

Huang, S.Y., Kuo, Y.H., Lee, W.C., Tsou, H.L., Lee, Y.P., Chang, H.L., Wu, J.J. and Yang, P.C. (1999). Substantial decrease of heat-shock protein HSP90 precedes the decline of sperm motility during cooling of boar spermatozoa. Theriogenology, 51. 1007-1016.

Isachenko, V., Soler, C., Isachenko, E., Perez-Sanchez, F. and Grishchenko, V. (1998). Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. Cryobiology 36. 250–253.

Ishimori, H, Takahashi, Y. and Kanagawa, H. (1992). Viability of Vitrified Mouse Embryos Using Various Cryoprotectant Mixtures. Theriogenology 37. 21-24.

Jaenicke, R. (1991). Protein stability and molecular adaptation to extreme conditions. Eur J Biochem 202. 715-728.

Kalichevski, M.T., Knorr, D. and Lillford, P.J. (1995). Potential application of high pressure effects on ice-water transitions. Trends Food Sci, Tech. 6. 253-259.

Kanno, H., Speedy, R.J. and Angell, C.A. (1975). Supercooling of water to -92 C under pressure. Science, 189. 880-881.

Kasai, M., Niwa, K. and Iritani, A. (1981). Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos. J. Reprod. Fertil. 63. 175-180.

Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. and Machida, T. (1990). A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil 89. 91-97.

Kashiwazaki, N., Ohtani, S., Miyamoto, K. and Ogava S. (1991). Production of normal piglets from hatched blastocysts frozen at -196 C. The Veterinary Record 128, 256-257.

Keskintepe, L. and Brackett, B.G. (2000). Cryopreservation of bovine blastocysts obtained by intracitoplasmatic sperm injection. Theriogenology 53. 1041-1052.

Khorram, O., Saphiro, S.S. and Jones, J.M. (2000). Transfer of non-assisted hatched and hatching human blastocysts after in vitro fertilization. Fertility and Sterility 74. 163-165.

Kong, I.K., Lee, S.I., Cho, S.G., Cho, S.K. and Park, C.S. (2000). Comparison of open pulled straw (OPS) vs Glass micropipette (GMP) vitrification in mouse blastocysts. Theriogenology, 53. 1817-1826.

Kuleshova, L.L., Shaw, J.M. and Trounson, A.O. (2001). Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. Cryobiology 43. 21-31.

Landa, V. (1982). A simple method for freezing mouse blastocysts. Folia Biologica 28, 266-273.

Landa, V. and Tepla, O. (1990). Cryopreservation of mouse 8-cell embryos in microdrops. Folia Biol. Praha 36. 153–158.

Lane, M., Forest, K.T., Lyons, E.A. and Bavister, B.D. (1999). Live births following vitrification of hamster embryos using a novel container-less technique. Theriogenology 51. 167.

Lassalle, B., Testart, J. and Renard, J.P. (1985). Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. Fertil. Steril. 44. 645-651.

LaTena, A., Brandi, A., Falconi, M., Spurio, R., Pon, C.L. and Gualerzi, C.O. (1991). Identification of a cold-shock transcriptional enhancer of the Escherichia coli major cold shock gene encoding nucleotide protein H-NS. Proc Natl Acad Sci USA 88.10907-10911.

Lehn-Jensen, H., Rall, H. and Willadsen, S.M. (1982). Direct observation of cattle bstocysts during freezng and thawing. Anim. Breeding Abstr. 51. 178.

Leibo, S.P. (1981). Preservation of ova and embryos by freezing. In: New technologies in animal breeding. (Eds.: Brackett, B.G., Seidel, G.E.Jr. and Seidel, S.M.) 127-139. Academic Press, London.

Leibo, S.P. (1982). A one-step method for direct non-surgical transfer of frozen-thawed bovine embryos. Int. Congr. Embryo Transf. Mammals 97.

Leibo, S.P. (1984). A one-step method for direct non-surgical transfer of frozen-thawed bovine embryos. Theriogenology 21. 767-790.

Leibo, S.P. (1986). Cryobiology: Preservation of mammalian embryos. In: Genetic engineering of animals. An agricultural perspective. 251-272. (Eds.: Warren, E.J. and Hollander, A.) Plenum Press, New York

Leibo, S.P. (1989). Equilibrium and nonequilibrium cryopreservation of embryos. Theriogenology 31. 85-93.

Leibo, S.P. and Songsasen, N. (2002). Cryopreservation of gamets and embryos of non-domestic species. Theriogenology 57. 303-326.

Leibo, S.P., Martino, A., Kobayashi, S. and Pollard, J.W. (1996). Stage-dependent sensitivity of oocytes and embryos to low temperatures. Anim Reprod Sci 42. 45-53.

Lewis, I.M., Lane, M.W. and Vajta, G., (1999). Pregnancy rates following transfer of in vitro produced bovine embryos vitrified by the Open Pulled Straw OPS method. Theriogenology 51. 168.

Li, R., Cameron, A.W.N., Batt, P.A. and Trounson, A.O. (1990). Maximum survival of frozen goat embryos is attained at the expanded, hatching and hatched blastocyst stages of development. Reprod. Fertil. Dev. 2. 345-350.

Liebermann, J., Nawroth, F., Isachenko, V., Isachenko, E., Rahimi, G. and Tucker, M.J. (2002). Potential importance of vitrification in reproductive medicine. Biol. Reprod. 67. 1671-1680.

Lopez-Bejar, M. and Lopez-Gatius, F. (2002). Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure. Theriogenology 58. 1541-1552.

Macdonald, A.G. (1987). in Current Perspectives in High Pressure Biology (eds Jonnasch, H.W., Marquis, R.E. and Zimmerman,A.M.) Academic, London, 207-223.

Macdonald, A.G. (1997). Hydrostatic pressure as an environmental factor in life processes. Comp. Biochem. Physiol. 116. 291-297.

MacFarlane, D.R. (1987). Physical aspects of vitrification in aqueous solutions. Cryobiology 24. 181-195.

MacFarlane, D.R., Angell, C.A. and Fahy, G.M. (1981). Homogenous nucleation and glass formation in cryoprotective systems at high pressures. Cryo-lett. 2. 353-358.

MacFarlane, D.R., Scheirer, J. and Smedley, S.I. (1986). Pressure coeffitient of conductance and of glass transition temperatures in concentrated aqueous LiCl, LiI and $AlCl_3$ solutions. J. Phys. Chem. 90. 2168-2173.

Martino, A., Pollard, J.A. and Leibo, S.P. (1996). Effect of chilling bovine oocytes on their developmental competence. Mol. Reprod. Dev. 45. 503–512.

Massip, A. and Van Der Zwalmen, P. (1984). Direct transfer of frozen cow embryos in glycerol-sucrose. Vet. Rec. 115. 327.

Massip, A., Van Der Zwalmen, P., Ectors, F., De Coster, R., D'Ieteren, C. and Hazen, C. (1979). Deep freezing of cattle embryos in glass ampoules and french straws. Theriogenology 12. 79-83.

Massip, A., Van Der Zwalmen, P., Scheffen, B. and Ectors, F. (1986). Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo. Lett. 7. 270-273.

Massip, A., Van Der Zwalmen, P. and Ectors, F. (1987). Recent progress in cryopreservation of cattle embryos. Theriogenology 27. 69.

Mauer, R.R. (1978). Freezing mammalian embryos: a review of techniques. Theriogenology 9. 45.

Mazur, P. (1980). Fundamental aspects of the freezing of cells, with emphasis on mammalian ova and embryos. Proc. 9th Int. Conr. Anim. Reprod. And A.I. 1. 99.

Mazur, P., Rigopoulos, N., Jackowski, S.C. and Leibo, S.P. (1976). Preliminary estimates of the permeability of mouse ova and early embryos to glycerol. Biophysical Journal 16, 232A.

Mazur, P. and Schneider, U. (1986). Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications. Cell Biophys. 8. 259-284.

Miyamoto, H. and Ishibashi, T. (1977). Survival of frozen-thawed mouse and rat embryos in the presence of ethylene glycol. J Reprod Fertil 50. 373-375.

Miyamoto, H. and Ishibashi, T. (1986). Liquid nitrogen vapour freezing of mouse embryos. J. Reprod. Fertil. 78. 471-478.

Murakami,T.H. and Zimmerman, A.M. (1973). DNA syntheseis in Tetrahymena: a pressure study. Cytobios 7. 171-181.

Nagashima, H., Kashiwazaki, N., Ashman, R.J., Grupen, C.G. and Nottle, M.B. (1995). Cryopreservation of porcine embryos. Nature 374. 416.

Nagashima, H., Kashiwazaki, N., Ashman, R.J., Grupen, C.G., Seamark, R.F. and Nottle, M.B. (1994). Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. Biol Reprod. 5. 618-622.

Nagashima, H., Kato, Y., Yamakawa, H., Matsumoto, T. and Ogawa, S. (1989). Changes in freezing tolerance of pig blastocysts in peri-hatching stage. Jpn. J. Anim. Reprod. 35. 130-l 34.

Nguyen, B.X., Sotomaru, Y., Tani, T., Kato, Y. and Tsunoda, Y. (2000). Efficient cryopreservation of bovine blastocysts derived from nuclear transfer with somatic cells using partial dahydration and vitrification. Theriogenology 53. 1439-1448.

Niemann, H., Sacher, B., Schilling, E. and Schmit, D. (1982). Improvement of survival rates of ovine blastocysts with sucrose for glycerol dilution after fast freezing and thawing method. Theriogenology 17. 102.

Noma, S. and Hayakawa, I. (2003). Barotolerance of Staphylococcus aureus is increased by incubation at below 0 °C prior to hydrostatic pressure treatment. Internat J Food Microbiol 80. 261-264.

Nowshari, M.A. and Brem, G. (1998). Effect of cryoprotectants and their concentrations on post-thaw survival and development of expanded mouse blastocysts frozen by a simple rapid-freezing procedure. Theriogenology 50. 1001-1013.

O'Neill, L., Paynter, S.J. and Fuller, B.J. (1997). Vitrification of mature mouse oocytes: Improved results following addition of polyethylene glycol to a dimethyl sulfoxide solution. Cryobiology 34. 295-301.

Ouhibi, N., Sadler F.K., Teramura, D., Eaton, D.L., Larson, J. and Patton P.E. (2000). Blastocyst cryosurvival and pregnancy rate with or without assisted hatching. Fertility and Sterility 74. 52.

Palou, E., Lopez-Malo, A., Barbosa Canovas, G.V., Welti-Chanes, J. and Swanson, B.G. (1997). Kinetic analysis of Zygosaccharomyces bailii inactivation by high hydrostatic pressure. Lebensm.-Wiss. U. Technol. 30. 703-708.

Papis, K., Shimizu, M. and Izaike, Y., (1999). The effect of gentle pre-equilibration on survival and development rates of bovine in vitro matured oocytes vitrified in droplets. Theriogenology 51. 173.

Parks, J.E. (1997). Hypothermia and Mammalian Gametes. In: Karow, AM, Critser, JK (eds.),Reproductive Tissue Banking: Scientific Principles. San Diego: Academic Press 229-261.

Parks, J.E. and Rufting, N.A. (1992). Factors affecting low temperature survival of mammalian oocytes.Theriogenology 37. 59-73.

Péqueux A. and Gilles, R. (1978). Effects of high hydrostatic pressures on the activity of the membrane ATPases of some organs implicated in hydromineral regulation. Comp Biochem Physiol B Biochem Mol Biol 59. 207-212.

Pettit, W.H.Jr. (1985). Commercial freezing of bovine embryos in glass ampules. Theriogenology 23. 13.

Phadtare, S., Alasina, J. and Inouye, M.. (1999). Cold-shock response and cold-shock proteins. Curr Opin Microbiol 2.175-180.

Polge, C., Smith, A.U. and Parks, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164. 666.

Pope, C.E., McRae, M.A., Plair, B.L., Keller, G.L. and Dresser, B.L. (1994). Successful in vitro and in vivo development of in vitro fertilized two- to four-cell cat embryos following cryopreservation, culture and transfer. Theriogenology 42. 513-525.

Pope, C.E., McRae, M.A., Plair, B.L., Keller, G.L. and Dresser, B.L. (1997). In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization of cat oocytes. J. Reprod. Fertil. 51. 69-82.

Pope, C.E., Pope, V.Z. and Beck, L.R. (1984). Live birth following cryopreservation and transfer of a baboon embryo. Fertil. Steril. 42. 143-145.

Quintans, C.J., Donaldson, M.J., Marazzi, A.N. and Pasqualini, S. (2001). Pregnancy and delivery following the transfer of blastocysts cryopreserved after hatching in propanediol/sucrose. Fertility and Sterility 76, 122.

Rall, W.F. (1987). Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology 24. 387–402.

Rall, W.F. (2001). Cryopreservation of mammalian embryos, gametes and ovarian tissues: current issues and progress. In: Assisted Fertilization and Nuclear Transfer in Mammals. (Eds.: Wolf, DP and Zelinski-Wooten, M), Humana Press, Totawa, NJ 173-187.

Rall, W.F. and Fahy, G.M. (1985). Ice-free cryopreservation of mouse embryos at -196 °C by vitrification. Nature 313. 573-575.

Raymond, C. (1997). Chemistry. 6th edition. Mcgraw-Hill, London

Reichenbach, H.D. and Rodrigues, J.L. (1988). Survival of mouse morulae and early blastocysts after direct plunging into liquid nitrogen. Theriogenology 29. 46-47.

Riha, J., Landa, V., Kneissl, J., Matus, J., Jindra, J. and Kloucek, Z. (1991). Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after nonsurgical transfer. Zivoc. Vir. 36. 113–120.

Ritz, M., Tholozan, J. L., Federighi, M. and Pilet, M. F. (2002). Physiological damages of Listeria monocytogenes treated by high hydrostatic pressure. Internat J Food Microbiol 79. 47-53.

Routray, P., Suzuki, T., Strüssmann, C.A. and Takai, R. (2002). Factors affecting the uptake of DMSO by the eggs and embryos of medaka, Oryzias latipes. Theriogenology 58. 1483-1496.

Schneider, U. (1986). Cryobiological princples of embryo freezing. J. In vitro Fertil. Embryo Transfer 3. 3-9.

Schneider, U. and Mazur, P. (1984). Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. Theriogenology 21. 68.

Schneider, U. and Mazur, P. (1986). Implications and applications of the long -term preservation of embryos by freezing. In: Current therapy in theriogenology Vol.2. (Ed.:Morrow, D. A.). Saunders, Philadelphia

Schmid, G., Lüdemann, H. D. and Jaenicke, R. (1975). High pressure effects on the activity of glycolytic enzymes. Biophys Chem 3. 90-98.

Schuster, B. and Sleytr, U. B. (2002). The effect of hydrostatic pressure on S-layer-supported lipid membranes. Biochim Biophys Acta 1563. 29-34.

Seki, K. and Toyoshima, M. (1998). Preserving tardigrades under pressure. Nature 395. 853-854.

Shaw, J.M., Diotallevi, L. and Trounson, A.O. (1991). A simple rapid 4.5 M dimethyl-sulfoxide freezing technique for the cryopreservation of one-cell to blastocyst stage praeimplantation mouse embryos. Reproduction Fertility and Development 3, 621-626.

Shaw, J.M., Kuleshova, L.L., MacFarlane, D.R. and Trounson, A.O. (1997). Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran. Cryobiology 35. 219-229.

Shaw, J.M., Ward, C. and Trounson, A.O. (1995). Survival of mouse blastocysts slow cooled in propanediol or ethylene glycol is influenced by the thawing procedure, sucrose and antifreeze proteins. Theriogenology 43, 1289-1300.

Silva, J. L., Foguel, D. and Royer, C. A. (2001). Pressure provides new insights into protein folding, dynamics and structure. Trends Biochem Sci 26. 612-618.

Skidmore, J.A., Boyle, M.S. and Allen, W.R. (1991). A comparison of two different methods of freezing horse embryos. J. Reprod. Fert. 44. 714-716.

Solti, L., Crichton, E.G., Loskutoff, N.M. and Cseh, S. (2001). Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. Theriogenology 53. 149-162.

Solti, L., Safaa Al-Hasani, Schneider, U. and Hahn, J. (1981). Mélyhűtési kísérletek nyúlembrióval. Magy. Áo. Lapja, 36. 758.

Sommerfeld, V. and Niemann, H. (1999). Cryopreservation of bovine in vitro produced embryos using ethylene glycol in controlled freezing or vitrification. Cryobiology 38. 95-105.

Sonoike, K., Setoyama, T., Kuma, Y., Shinno, T., Fukumoto, K. and Ishihara, M. (1993). in High Pressure Bioscience and Food Science (ed Hayashi, R.) Sanei, Kyoto 213–219.

Spilimbergo, S., Elvassore, N. and Bertucco. A. (2002). Microbial inactivation by high-pressure. The Journal of Supercritical Fluids 22. 55-63.

Steponkus, P.L., Myers, S.P., Lynch, D.V., Gardner, L., Bronshteyn, V., Leibo, S.P., Rall, W.F., Pitt, R.E., Lin, T.-T. and MacIntyre, R.J. (1990). Cryopreservation of Drosophila melanogaster embryos. Nature 345. 170–172.

Steponkus, P.L., Stout, D.G., Wolfe, J. and Lovelace, R.V.E. (1985). Possible role of trasient electric fields in freezing-induced nenbrane destabilisation. J. Memb. Biol. 85. 191-198.

Summers, P.M., Shephard, A.M., Taylor, C.T. and Hearn, J.P. (1987). The effects of cryopreservation and transfer on embryonic development in the common marmoset monkey, Callithrix jacchus. J. Reprod. Fertil. 79. 241-250.

Takahashi, T., Kakita, A., Takahashi, Y., Sakamoto, I., Yokoyama, K., Fujiu, T., Yamashina, S., Tamaki, T., Takazawa Y. and Muratsubaki, R. (2000). Functional integrity of the rat liver after subzero preservation under high pressure. Transplant. Proc. 32. 1634-1636

Takahashi, T., Kakita, A., Takahashi, Y., Yokoyama, K., Sakamoto, I. and Yamashina, S. (2001). Preservation of rat livers by supercooling under high pressure. Transplant. Proc. 33. 916-919.

Takahashi, Y. and Kanagawa, H. (1990). Effect of equilibration period on the viability of frozen-thawed mouse moulae after rapid freezing. Mol. Reprod. Dev. 26. 105-110.

Takeda, T., Elsden, R.P. and Seidel, G.E. (1984). Cryopreservation of mouse embryos by direct plunging into liquid nitrogen. Theriogenology 21. 266 abstr.

Tauc, P., Mateo, C. R. and Brochon, J. C. (2002). Investigation of the effect of high hydrostatic pressure on proteins and lipidic membranes by dynamic fluorescence spectroscopy. Biochim Biophys Acta 1595. 103-115.

Trounson, A.O. and Mohr, L. (1983). Human pregnancy following cryopreservation, thawing and transfer of eight-cell embryo. Nature 305. 707.

Tsunoda, Y., Soma, T. and Sugie, T. (1982). Effect of post-ovulatory age of recipient on survivalof frozen-thawed rabbit morulae. Journal of Reproduction and Fertility 65, 483-487.

Vajta, G., Booth, P.J., Holm, P., Greve, T. and Callesen, H., (1997). Successful vitrification of early stage bovine in vitro produced embryos with the Open Pulled Straw OPS method. Cryo-Lett. 18. 191–195.

Vajta, G., Murphy, C., Machaty, Z., Prather, R.S., Greve, T. and Callesen, H., (1999). In-straw dilution of in vitro produced bovine blastocysts after vitrification with the Open Pulled Straw OPS method. Vet. Rec. 144. 180–181.

van Wagtendonk-de Leeuw, A.M., den Daas J.H.G. and Rall, W.F. (1994). Pregnancy rates in a comperative field trial of vitrification and one-step dilution or conventional slow freezingand three-step dilution of bovine embryos are similar. Theriogenology 41. 326.

Weber, G. and Drickamer, H.G. (1983). The effect of high pressure upon proteins and other biomolecules. Q Rev Biophys 16. 89-112.

Welch, T. J., Farewell, A., Neidhardt, F. C., and Bartlett, D. H. (1993). Stress response of Escherichia coli to elevated hydrostatic pressure. J. Bacteriol. 175. 7170-7177.

Wemekamp-Kamphuis, H.H., Karatzas, A.K., Wouters, J.A. and Abee, T. (2002). Enhanced levels of cold shock proteins in Listeria monocytogenes LO28 upon exposure to low temperature and high hydrostatic pressure. Appl Environ Microbiol. 68. 456-63.

Wen-Lei, C., Yi-Xin, W., Zu-Qiong, X. and Zheng, L. (2003). Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism. Asian Journal Of Andrology 5. 43-46.

Whittingham, D.G. (1971). Survival of mouse embryos after freezing and thawing. Nature 233, 125-126.

Whittingham, D.G. (1975). Survival of rat embryos after freezing and thawing. J. Reprod. Fert. 43. 575.

Whittingham, D.G. (1979). In-vitro fertilization, embryo transfer and storage. Br. Med. Bull. 35. 105.

Whittingham, D.G., Leibo, S.P. and Mazur, P. (1972). Survival of mouse embryos frozen to – 196 °C and – 269 °C. Science 178. 411-414.

Willadsen, S.M. (1977). Factors affecting the survival of sheep embryos during deep-freezing and thawing. In: Ciba Found Symp 52. The Freezing of Mammalian Embryos., Elsevier, Amsterdam. 175-201.

Willadsen, S.M. (1980). Deep freezing of embryos of the large domestic species. Proc. 9th Int. Congr. Anim. Reprod. And A.I. 2. 255.

Willadsen, S.M., Polge, C., Rowson, L.E.A. and Moore, R.M. (1974). Preservation of sheep embryos in liquid nitrogen. Cryobiology 11. 560.

Willadsen, S.M., Polge, C., Rowson, L.E.A. and Moor, R.M. (1976). Deep freezing of sheep embryos. Journal of Reproduction and Fertility 46, 151-154.

Willet, E.L., Buckner, P.J. and Larson, G.L. (1953). Three successful transplantations of fertilized bovine eggs. J. Dairy Sci. 37. 520.

Wilmut, I. (1972). The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. Life Sci. 11. 1071.

Wilmut, I. and Rowson, L.E.A. (1973). Experiments on the low temperature preservation of cow embryos. Vet. Record 92. 686-690.

Wilson, P.W., Henegham, A.F. and Hazmet, A.D.J. (2003). Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation. Cryobiology 46. 88-98.

Wolfe, J., Dowgert, M.F. and Steponkus, P.L. (1986). mechanical study of deformation and rupture of plasma membranes of protoplasts during osmotic expansions. J. Memb. Biol. 93 . 63-74.

Wolfe, J. and Bryant, G. (1992). Physical principles of membrane damage due to dehydration and freezing. In: Mechanics of swelling: from clays to living cells and tissues. (Ed.:Karalis, T.K.) NATO ASI Series H. 64. 205-224.

Wolfe, J. and Bryant, G. (2001). Cellular cryobiology: thermodynamic and mechanical effects. International journal of refrigeration. 24. 438-450.

Wood, M.J. and Farrant, J. (1980). Preservation of mouse embryos by two-step freezing. Cryobiology 17. 178-180.

Wouters JA, Jeynov B, Rombouts FM, de Vos WM, Kuipers OP and Abee T. (1999). Analysis of the role of 7 kDa cold-shock proteins of Lactobacillus lactis MG1363 in cryoprotection. Microbiology 145. 3185-3194.

Wright, J.M. (1985). Commercial freezing of bovine embryos in straws. Theriogenology 23. 17.

Xu, K., Palasz, A., Moker, J. and Mapletoft, R.J. (1988). Fast freezing of mouse morulae. Theriogenology 29. 34-37.

Yager, P. and Chang, E. L. (1983). Destabilization of a lipid non-bilayer phase by high pressure. Biochim Biophys Acta 731. 491-494.

Yamamoto, Y., Oguri, N. and Hachimohe, Y. (1983). Viability of equine embryos after cooling to low temperatures. Proc. 5th World Conf. Anim. Prod. Tokyo 2. 20.

Yamamoto, Y., Oguri, N., Tsutsumi, Y. and Hachimohe, Y. (1982). Experiments in the freezing and storage of equine embryos. J. Reprod. Fertil. 32. 399.

Yamanaka K, Fang L. and Inouye, M. (1998). The CspA family in Escherichia coli: multiple gene duplication for stress adaptation. Mol Microbiol 27. 247-255.

Yang, B.S. and Leibo, S.P., (1999). Viability of in vitro-derived bovine zygotes cryopreserved in microdrops. Theriogenology 51. 178.

Yoon, H.G., Yoon, S.H., Son, W.Y., Im, K.S. and Lim, J.H. (2001). High implantation and pregnancy rates with transfer of human hatching day 6 blastocysts. Fertility and Sterility 75. 832-833.

Zenzes, M.T., Bielecki, R., Casper, R.F. and Leibo, S.P. (2000). Effects of chilling to 0°C on the morphology of meiotic spindles in human metaphase II oocytes. Fertil Steril 75. 769-777.

Zeron, Y., Pearl, M., Borochov, A. and Arav, A. (1999). Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. Cryobiology 38. 35–42.

Zhu, S.E., Sakurai, T., Edashige, T., Machida, T. and Kasai, M. (1996). Cryopreservation of zona-hatched mouse blastocysts. Journal of Reproduction and Fertility 107. 37-42.