SOME FACTORS AFFECTING SUCCESSFUL VITRIFICATION OF MOUSE BLASTOCYSTS

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ABSTRACT

The effects of temperature and exposure time to vitrification solutions on In vitro survival of mouse blastocysts were investigated. Blastocysts were first exposed for 10 min to vitrification Solution 1 (VS1) containing 10% glycerol-20% 1,2 propanediol in phosphate buffered saline (PBS), then to vitrification Solution 2 (VS2) with 25 % glycerol-25% 1,2 propanediol for various periods either at room temperature or at 4°C. At room temperature survival dropped quickly, while at 4°C an increase in survival was observed.

It is concluded that the viability of mouse blastocyts after vitrification is dependent on the temperature and duration of equilibration in vitrification solutions.

Key words: mouse, blastocysts, cryopreservation, vitrification, cryoprotectants

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INTRODUCTION

Vitrification is a new approach for cryopreservation of mammalian embryos. Optimization of the quick freezing of mammalian embryos by using vitrification solutions was first proposed by Rall and Fahy (1) and Rall and al.(2).They reported in vitro and in vivo survival of eight-cell mouse embryos. Their vitrification medium was composed of three permeating solutes (dimethylsulfoxyde, acetamide, propanediol) and a non-penetrating polymer (polyethylene glycol) in PBS. This vitrification solution is rather toxic and thus requires manipulation of embryos at different temperatures (ambient and 4°C) for various times to different cryoprotectant levels before direct transfer to liquid nitrogen.

Scheffen and al. (3) developed a vitrification procedure for Day 4 mouse embryos involving a mixture of glycerol and 1,2 propanediol in different proportions of PBS: 10% glycerol-20% propanediol as equilibration medium at ambient temperature for 10 min followed by a short equilibration time (maximum 30 sec) at ambient temperature in an extracellular vitrification solution consisting of 25% glycerol-25% 1,2 propanediol before plunging in liquid nitrogen (LN2). Using this method, in vitro survival was lower for blastocysts than for compacted morulae and early blastocysts (39% vs 80%).

We have conducted some experiments to investigate the effect of temperature and exposure time to vitrification solutions on in vitro survival of blastocysts.

MATERIALS AND METHODS

Embryos were recovered from superovulated Swiss albino OF1 mice on Day 4 (Day 1 = day of presence of the copulation plug) by flushing the uterine horns with PBS. After embryo classification, only blastocysts were retained while morulae and early blastocysts were discarded .

Two vitrification solutions were prepared as described previously(3). The first (VS1) was a mixture of 10 % glycerol-20 % 1,2 propanediol in PBS (V/V). The second solution (VS2) contained 25 % glycerol-25 % 1,2 propanediol (V/V).

The vitrification procedure has been described elsewhere (3 ,4)..In brief, embryos were exposed to VS1 for 10 min at ambient temperature (Experiments 1and 2) or at 4°C (Experiment 3). Then they were loaded into a small column of VS2 (± 20µl) in .25 ml transparent straws[a] separe-

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ted by two air bubbles from the rest of the straw filled with 1 M sucrose solution (1/10-12). After different equilibration periods in VS2, a portion of the straws were emptied directly into a watch glass and left at room temperature before being washed in PBS and cultured. The remaining straws were plunged directly in liquid nitrogen and then stored.

Thawing was carried out by gently shaking the straw in a water bath at 20°C until ice had disappeared in the sucrose solution. The contents of the straw were emptied into a watch glass and left for 5 min at room temperature. The embryos were then washed in PBS and cultured.

Embryo survival following treatment was determined by the percentage of blastocysts that expanded again during a 24 h culture period. Embryos were cultured in Falcon tubes (12 x 75 mm) containing 1 ml T6 medium (5) with 20 % fetal calf serum under dimethyl-polysiloxane oil (DMPS).

Statistical analysis was carried out with χ^2 tests.

Three experiments were conducted to investigate the effects of temperature and exposure time of mouse blastocysts to cryoprotective solutions during cryopreservation by a modified vitrification procedure The experimental procedure is described in table 1.

Table 1. Outline of the experimental procedure

	Experiment No					
	1		2		3	
	VS1	VS2	VS1	VS2	VS1	VS2
Temperature, (°C)	AT[a]	AT[a]	AT[a]	4°C	4°C	4°C
Time (minutes)	10	0.5-30[b]	10	0.5-20[b]	10	0.5-120[c]

a Ambient temperature.

b 5min increments.

c 10min increments.

RESULTS

Survival of both equilibrated and vitrified blastocysts dropped dramatically for 5min exposure time when equilibrated in VS2 at room temperature (Figure 1).

On the other hand, when VS2 was precooled and maintained at 4°C as in Experiment 2, the survival of vitrified blastocysts was greatly improved (72% vs 43% at 5 min, $P<0.001$; Figure 2), but it also decreased with time spent in VS2 (40% after 10 min).

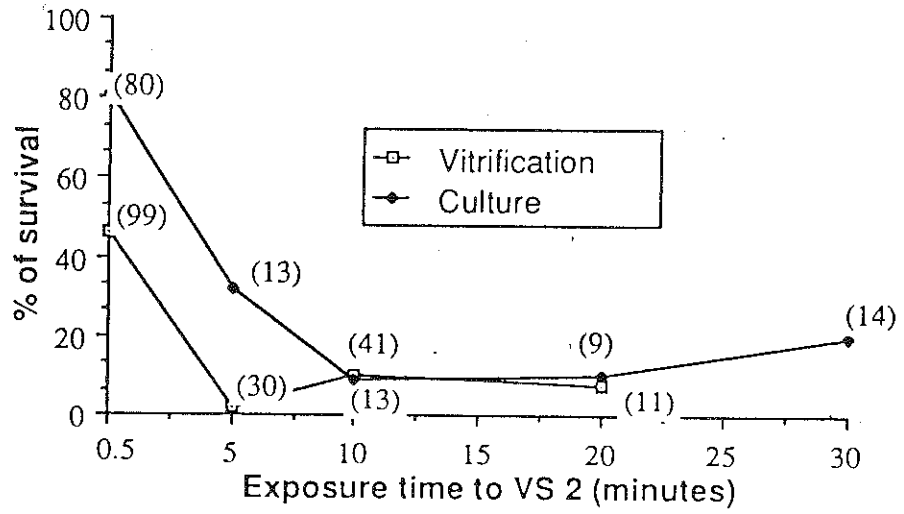

Figure 1: Survival of mouse blastocysts after a 10 minutes exposure to vitrification solution 1 and various time periods to VS2 both at room temperature followed by culture or vitrification plus culture. ( ) = number of embryos treated.

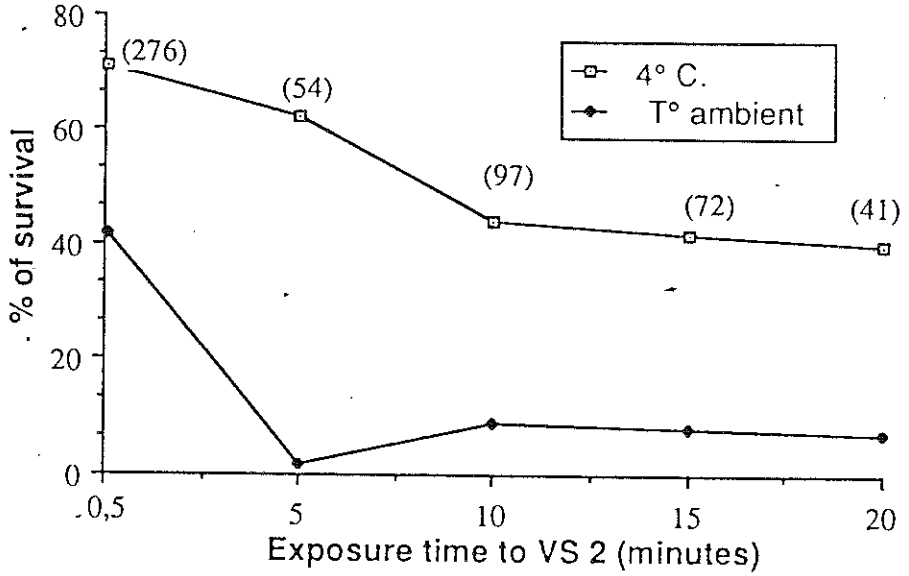

Figure 2 : Survival of mouse blastocysts after a 10 min exposure to VS 1 at room temperature and various time periods to VS 2 either at for 4°C or at room T° followed by vitrification.

When the two steps were performed at 4°C, survival rate following equilibration was very high (≥ 80%) only from 0 to 80 min, while following vitrification, it increased sharply to a maximum rate (70 to 80 %) between 20 and 80 min (figure 3).

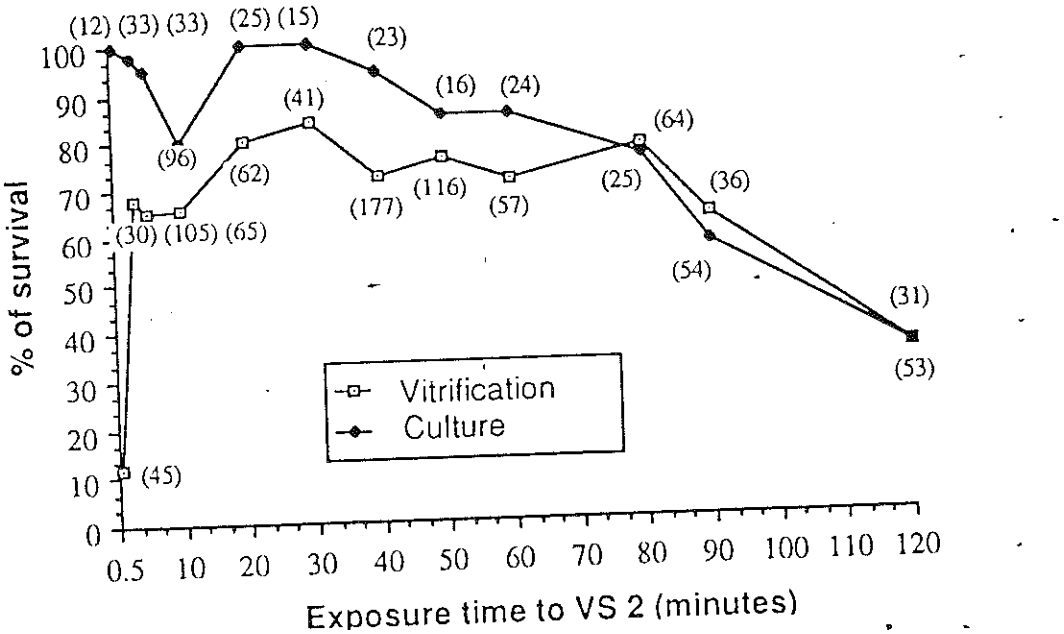

Figure 3 : Survival of mouse blastocysts after a 10 min exposure to VS 1 and various time period to VS 2 at 4 °C followed by culture or vitrification.

DISCUSSION

The blastocyst is more complex than the morula in that it contains an inner cell mass, a trophoblastic layer and a blastocoelic cavity. Thus different cell types exist and different metabolic activity and permeability probably occur compared with earlier stages of development. There is experimental proof in mice that the permeability coefficient for glycerol increases from the onecell to the blastocyst stage (6,7). With a high permeability coefficient, we can expect few osmotic problems, particularly at room temperature, since the permeability coefficient is temperature dependent (8).

Therefore, at ambiant temperature the sharp decrease in viability (Experiment 1, Figure 1) for blastocysts, both equilibrated and vitrified, should be attributed to chemical toxicity rather than to osmotic injury. In

fact, when permeability was increased by equilibration at 37°C with VS2 only, no survival was evident (personal observation).

When blastocysts were equilibrated in VS2 at 4°C for 0.5 min (Experiment 2, Figure 2), the efflux of water and the shrinkage of the blastocyst was followed by a slow influx of VS2, leading to a partial dehydration of the blastocyst and a consequent increase in concentration of cryoprotectants that, when associated with cytoplasmic proteins (1),favors vitrification even without full equilibration in VS2.

Exposure of embryos to hight osmotic pressures created an osmotic pressure gradient between the intra- and extra-cellular space which, if it exceeds a tolerable limit, may damage the cell membrane (10,12). This may explain the decrease in survival for longer equilibration times in VS2 at 4°C.

Indeed the unequilibrated situation increases the intracellular concentration (VS1) to approximately 30%; a concentration shown previously (3) to be toxic to blastocysts. In this hypothesis, decreasing the dehydration osmotic pressure improves the survival rate. This was observed by performing the two steps at 4°C, optimal intracellular vitrification conditions were reached for equilibration times in VS2 ranging between 20 and 80 min. .In these conditions, when the blastocysts were equilibrated in VS2, the lower efflux of water induced a lower concentration of intacellular VS1 than that obtained at room temperature.

The results of these experiments demonstrate that the survival of vitrified mouse blastocysts is dependent on temperature and duration of equilibration in vitrification solutions and augments previous reports on the use of embryos frozen in glycerol-sucrose solution by a rapid freezing method(11,12).

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