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Cryopreservation reduces the ability of hamster 2-cell embryos to regulate intracellular pH

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Vitrification of hamster 2-cell embryos impairs the activity of both the Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger; the two transport proteins responsible for the regulation of intracellular pH (pHi). The activities of both the Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger were significantly reduced at 4 h following warming compared to freshly collected embryos. Normal levels of activity of both transporters were not restored until 6 h after warming. Thus, cryopreservation of cleavage stage hamster embryos has a detrimental effect on their ability to maintain intracellular ionic homeostasis. Impairment of these pHi regulatory proteins resulted in the pHi of embryos being significantly elevated from the control values of 1.2 to 7.35 for approximately 4 h after warming. In addition, an elevated pHi value significantly impaired oxidative metabolism. Therefore, the loss in developmental competence of embryos following cryopreservation may in part be explained by a reduced ability to regulate intracellular pH that results in perturbations in metabolism and disruption of energy production.

Key words: cryopreservation/HCO_3^-/Cl^- transporter/metabolism/Na^+/H^+ antiporter/vitrification

Introduction

Cryopreservation is an important aspect of assisted reproductive technologies as it allows embryos to be stored indefinitely until replacement into the mother. While it is possible to cryopreserve embryos from several species, the efficiency is low, as evidenced by observations that cryopreserved embryos have a reduced capacity to both establish and maintain a pregnancy (Ménézo and Veiga, 1997). It has recently been demonstrated that the ability of cleavage stage embryos to maintain developmental competence in culture is related to their ability to regulate intracellular pH (LeClerc *et al*., 1994; Zhao *et al*., 1995; Lane *et al*., 1998, 1999a). Intracellular pH (pHi) plays a key regulatory role in many cellular processes such as metabolism, energy production, and cell division (Begg and Rebhun, 1979; Regula *et al*., 1981). Hamster embryos regulate pHi primarily by the activity of two transporters located in the cell membrane: the Na^+/H^+ antiporter and the HCO_3^-/Cl^- exchanger (Lane *et al*., 1998, 1999a). The Na^+/H^+ antiporter regulates pHi in the acid to neutral range and the HCO_3^-/Cl^- transporter regulates pHi in the neutral to alkaline range. Inhibiting these transporters results in an inability of hamster 2-cell embryos to regulate pHi and subsequent development in culture is reduced. The effect of cryopreservation on the homeostatic mechanisms in embryos is currently unknown. However, it is possible that loss of developmental competence following cryopreservation of embryos is due to disruptions in mechanisms of ionic homeostasis, particularly in pHi. Prolonged exposure of mouse zygotes to propanediol for 2–7 min has been shown to reduce pHi (Damien *et al*., 1990) Therefore, the aim of this study was to examine the regulation of pHi transport systems in hamster embryos following cryopreservation by vitrification.

Materials and methods

Media

Medium used for embryo culture was hamster embryo culture medium-10 (HECM-10; Table 1). For embryo collection and cryopreservation a HEPES-buffered HECM-10 (H-H10) was used (Table I). Medium for assessment of Na^+/H^+ antiporter activity was a bicarbonate-free modification of H-H10 supplemented with 0.5 mmol/l taurine and with a reduced lactate concentration (bfHH3t, Table 1). Medium for assessment of HCO_3^-/Cl^- transporter activity was a bicarbonate-buffered medium containing taurine and a reduced lactate concentration (H3t; Table 1). All media components and reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

Animals

Two-cell embryos were collected from 3–4 month old golden hamster females. Multiple ovulations were induced by an i.p. injection of pregnant mare's serum gonadotrophin (PMSG, Pregnyl, Diosynth, Chicago, IL, USA) on the day of post-oestrous. Females were mated to males on day 4 of the oestrous cycle and 2-cell embryos were collected between 30 and 32 h post-egg activation by spermatozoa (Bavister *et al*., 1983). Embryos were flushed from the oviduct with medium H-H10 at 37°C.

Open pulled straw vitrification

Two-cell hamster embryos were vitrified using the open-pulled straw (OPS) technique as described (Vajta et al. 1997; Lane *et al*., 1999b). French straws (0.5 ml; Minitube, Verona, WI) were pulled to twice the original length over a flame then cut in half and the plugged end removed. Two-cell embryos were incubated for 2 min in 10% ethylene glycol and 10% dimethylsulphoxide (DMSO) and then for between 20–30 s in 20% ethylene glycol, 20% DMSO, and 0.67 mol/l sucrose. Ten to 12 embryos were pipetted into a 1 µl drop of the second

Table I. Composition of media (mmol/l) used in the study

Component	HECM-10	H-H10*[b]	bfHH3t[b]	H3t
NaCl	113.8	113.8	113.8	113.8
KCl	3.0	3.0	3.0	3.0
$MgCl_2.6H_2O$	2.0	2.0	0.5	0.5
$CaCl_2.2H_2O$	1.0	1.0	1.0	1.0
$NaHCO_3$	25.0	5.0	–	25.0
Sodium lactate[#]	4.5	1.0	1.0	1.0
Glutamine	0.5	0.5	0.5	0.5
Taurine	0.5	0.5	0.5	0.5
Amino acids (each at)[+]	0.1	–	–	–
HEPES	–	20.0	25.0	–
Polyvinyl alcohol (PVA)	–	0.1 mg/ml	–	–

[b]Media were adjusted to pH 7.35 with NaOH; [#]concentration of L-isomer (sodium lactate was added as 50% D/L isomers); *for cryopreservation this medium contained 4 mg/ml bovine serum albumin (BSA) instead of PVA; [+]amino acids present are asparagine, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, serine; HECM-10 = hamster embryo culture medium-10; H-H10 = HEPES-buffered HECM-10; bfHH3t = bicarbonate-free H-H10, supplemented with 0.5 mmol/l taurine and reduced lactate concentration; H3t = bicarbonate-buffered medium containing taurine and reduced lactate concentration.

cryopreservation solution and then loaded into a pulled straw using capillary action. The straw containing the embryos was plunged directly into liquid nitrogen. Embryos were stored in liquid nitrogen for between 1 and 21 days. For warming, embryos were expelled from the straw in H-H10 containing 0.25 mol/l sucrose by the pressure from warming of the medium in the straw. Embryos remained in this medium for 5 min before transfer to H-H10 medium containing 0.125 mol/l sucrose for a additional 5 min. Embryos were washed twice more in medium H-H10 for 5 min each and then either placed in culture or assessed for intracellular pH regulation. This OPS procedure has previously been successful for cryopreserving hamster 2-cell embryos, resulting in morula/blastocyst development rates of around 50% and in normal fetal development after transfer (Lane *et al*., 1999b)

Embryo culture

Embryos were cultured in 35 µl drops of medium HECM-10 in groups of 10–12 under mineral oil (Sigma Chemical Co.) at 37°C in a humidified atmosphere of 10% CO_2, 5% O_2 and 85% N_2.

Measurement of intracellular pH

Intracellular pH was measured using the pH sensitive probe 2′, 7′-Bis (2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF; Molecular Probes, Eugene, OR, USA). Two-cell embryos were loaded with 0.7 µmol/l BCECF using the acetoxymethyl ester (BCECF-AM) for 20 min at 37°C in bfHH3t. Embryos were washed twice in medium without the probe and 8–12 embryos placed in a temperature-controlled chamber (Biophysica, Baltimore, MD, USA) at 37°C. For assessment of HCO_3^-/Cl^- transporter activity the chamber was gassed with 10% CO_2, 5% O_2, and 85% N_2. Measurement of pHi was achieved using a Nikon Diaphot inverted microscope connected by a Nikon Dual Optical Path Tube to a Photometrics PXL cooled camera (Huntington Beach, CA, USA) for high resolution recording of epifluorescent images. Analysis of fluorescent images was performed using Metamorph/Metafluor hardware and software (Universal Imaging Corporation, West Chester, PA, USA). Emission wavelength was set to 530 nm and the ratio of fluorescence intensities of excitation wavelengths 500 (pH sensitive) to 450 nm (pH insensitive) was obtained for each embryo. Fluorescent ratios were calibrated in situ using a nigericin/high K^+ method at four pH values: 6.7, 7.0, 7.4 and 7.8 for acid-loading and 7.0, 7.3, 7.7 and 8.1 for alkaline loading. The ratio of fluorescence intensity was linearly proportional to pH ($r^2 = 0.972$).

Determination of recovery from acidosis

Intracellular acidosis was induced by a NH_4Cl pulse after measurement of baseline pHi. Embryos were incubated for 10 min with 25 mmol/l NH_4Cl, which resulted in an immediate alkalization due to rapid equilibration of NH_3 across the membrane. A slower movement of NH_4^+ results in a slow acidification. Subsequent removal of the NH_4Cl from the medium causes acidification as the NH_3 leaves the cell rapidly, leaving behind the H^+ which entered the cell as NH_4^+ (Boron and DeWeer, 1976; Roos and Boron, 1981). Recovery was assessed for 20 min following removal of $NH_4^+$and calculated by determining the gradient of a tangent to the recovery curve (Baltz *et al*., 1990; Lane *et al*., 1998).

Determination of recovery from alkalosis

Following measurement of baseline pHi, the chamber was flushed with Cl^--free medium for 5–7 min which, due to the reverse activity of the HCO_3^-/Cl^- exchanger, causes a small increase in pHi. A further larger intracellular alkaline load was induced by incubation with 25 mmol/l NH_4Cl in medium containing Cl^-. This produces an immediate increase in pHi. Recovery from alkalosis was assessed for a subsequent 20 min. Recovery was calculated by determining the gradient of the tangent to the recovery curve (Baltz *et al*., 1991; Zhao *et al*., 1995; Lane *et al*., 1999a).

Assessment of embryo metabolism

Glycolytic activity and oxidative metabolism of 2-cell embryos were determined by incubation with the radioisotopes [5-^{3}H]-glucose and [U-^{14}C]-lactate respectively. Individual embryos were incubated in a 3 µl drop of medium HECM-10 containing 5.0 mmol/l [U-^{14}C]-lactate (0.025 µCi/µl) and 0.5 mmol/l glucose (0.002 µCi/µl) on the lid of a microcentrifuge tube over 1.5 ml of 25 mmol/l $NaHCO_3$ (O'Fallon and Wright, 1986; Rieger et al. 1992). The tubes were gassed with 10% CO_2: 5% O_2:85% N_2, sealed and incubated at 37°C for 3 h. Sham preparations of medium without embryos were included to control for non-specific breakdown of the label. Total radioactivity was determined by adding 3 µl of medium containing the label to the lid of a vial and inverting the tube to mix with the $NaHCO_3$ trap. After the 3 h incubation period, the tubes were opened and 1 ml of the $NaHCO_3$ trap was placed into a scintillation vial containing 200 µl of 0.1 mol/l NaOH, and the vials stored overnight at 4°C. The following day, 10 ml of scintillation fluid (Ultima Gold; Packard, Meriden, CT, USA) was added to each vial, the vials vortexed and counted for 4 min. Glycolytic activity and oxidative metabolism were calculated using the recovery efficiency of the radioisotope as previously described (Rieger *et al*., 1992).

Statistical analysis

Differences between treatments for pHi measurements and oxidative metabolism were assessed using analysis of variance followed by Bonferroni's multiple comparison procedure. Data for glycolytic activity was assessed using a non-parametric test due to the variances being significantly different between the treatments. Differences between treatments were therefore assessed using a Kruskall–Wallis test followed by a Dunn's test.

Results

Measurement of resting pH and buffering capacity of embryos following vitrification

The intracellular pH of control 2-cell embryos measured immediately following collection from the oviduct was

Table II. Recovery from acidosis by hamster embryos following cryopreservation

Time after warming (h)	Resting pHi	Buffering capacity (mmol/l/pH)	Recovery rate (pH units/min)	Final pHi after 20 min recovery time
Fresh, in-vivo	7.24 ± 0.01	21.76 ± 2.5	0.210 ± 0.038	7.15 ± 0.02
0	7.34 ± 0.03*	11.65 ± 2.2*	0.102 ± 0.002*	6.97 ± 0.03
2	7.38 ± 0.03*	16.62 ± 4.5	0.151 ± 0.027*	6.98 ± 0.03
4	7.34 ± 0.03*	18.15 ± 3.5	0.162 ± 0.021+	7.02 ± 0.01
6	7.25 ± 0.04	15.83 ± 3.1	0.210 ± 0.015	7.08 ± 0.02

n = at least 80 embryos (at least five replicates per time point).
* significantly different from control embryos ($P < 0.05$).
+ different from control embryos ($P = 0.08$).

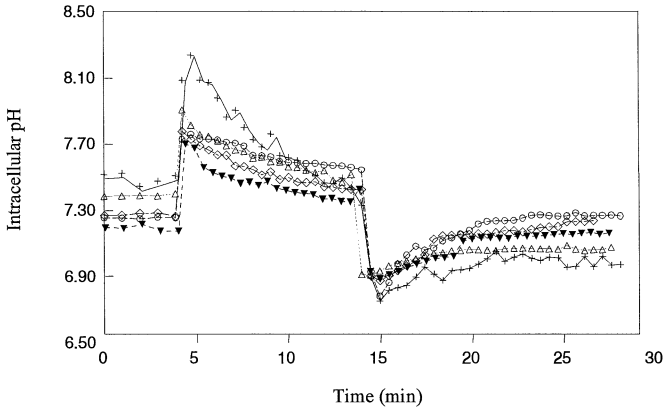

Figure 1. Recovery from acidosis by hamster 2-cell embryos after cryopreservation. Embryos were incubated in control medium bfHH3t for 5 min, before addition of 25 mmol/l NH_4Cl for 10 min. Intracellular acidosis was induced by removal of the NH_4Cl and recovery assessed in control medium bfHH3t. ▼ Control embryos measured immediately after collection; + embryos measured immediately following warming; △ embryos measured 2 h after warming; ○ embryos measured 4 h following warming; ◇ embryos measured 6 h after warming. For details of bfHH3t medium, see Table I.

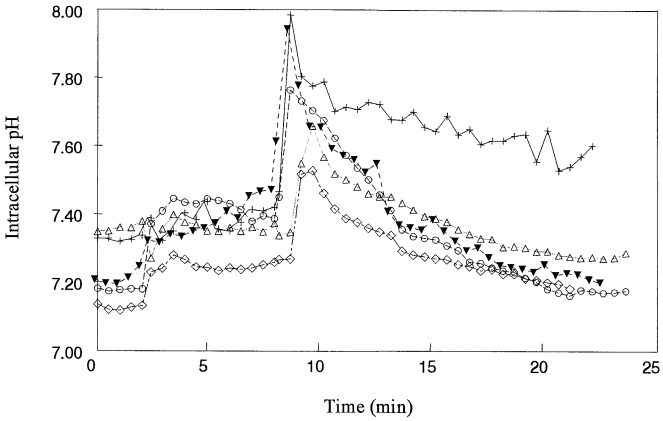

Figure 2. Recovery from alkalosis by hamster 2-cell embryos after cryopreservation. Embryos were incubated in control medium H3t before removal of Cl^- from the medium for 5–7 min. Intracellular alkalosis was induced by incubation with 25 mmol/l NH_4Cl and recovery assessed. ▼ Control embryos measured immediately following collection; + embryos measured immediately after warming; △ embryos measured 2 h following warming; ○ embryos measured 4 h after warming; ◇ embryos measured 6 h following warming. For details of H3t medium, see Table I.

7.24 ± 0.01. In contrast, embryos that were vitrified and assessed immediately following warming had a significantly increased baseline pHi value compared to that of the control embryos (Table II). The pHi of the treated embryos remained elevated until 6 h after warming.

The buffering capacity of embryos determines the ability of the cytoplasm to buffer changes in pHi (Baltz *et al*., 1991). The buffering capacity of 2-cell embryos was reduced immediately following cryopreservation compared to control embryos (Table II). However, by 2 h after warming the buffering capacity was similar to the control embryos (Table II).

Measurement of Na^+/H^+ antiporter activity after vitrification

Control 2-cell embryos recovered quickly from an induced acidosis (0.210 ± 0.08 pH units) and after the 20 min recovery period the pHi of the embryos had returned to that at the beginning of the incubation (Figure 1; Table II). In contrast, the rate of recovery from acidosis by vitrified 2-cell embryos measured immediately following warming was significantly reduced compared to freshly collected embryos (Table II). Consequently, at the end of the 20 min incubation period, the pHi that the embryos had re-established was significantly lower than the initial resting pHi (Table II). It also appeared that the rate of recovery had levelled off at this lower pHi (Figure 2). Of the nine replicates performed at this time point, only embryos from one replicate were able to restore pHi to that observed before acid loading.

Similarly, vitrified embryos assessed 2 h after warming had a significantly reduced rate of recovery from acidosis compared to the control embryos (Table II). Again these embryos were unable to restore pHi to normal values following acid-loading (Figure 1). Vitrified embryos that had been cultured for 4 h following warming had an increased rate of recovery from acidosis compared to embryos measured at 0 and 2 h post-warming (Table II). However, this recovery rate was reduced in comparison to that of freshly collected embryos ($P < 0.05$; Table II). By 6 h post-warming, there was no difference in the resting pHi of vitrified embryos at the beginning of the experiment or in the rate of recovery from acidosis compared to the control embryos (Table II). These embryos were able to recover pHi to values no different from the control embryos following acid loading (Table II; Figure 1).

Table III. Recovery from alkalosis by hamster embryos

Time after warming (h)	Increase in pHi after removal of CT^-	Rate of recovery (pH units/min)	Final pHi after 15 min recovery time
Fresh	0.211 ± 0.018	0.184 ± 0.012	7.22 ± 0.02
0	0.095 ± 0.021*	0.049 ± 0.009*	7.47 ± 0.04*
2	0.182 ± 0.048	0.062 ± 0.010*	7.39 ± 0.06*
4	0.171 ± 0.051	0.092 ± 0.008*	7.32 ± 0.03
6	0.196 ± 0.024	0.178 ± 0.011	7.30 ± 0.02

n = at least 60 embryos (at least four replicates per time point).
* significantly different from control embryos ($P < 0.05$).

Determination of HCO_3^-/Cl^- exchanger activity following vitrification

Removing Cl^- from the medium for freshly collected 2-cell embryos resulted in an increase in pHi of around 0.2 pH units. These control embryos also exhibited high rates of recovery from alkalosis and at the end of the 15 min incubation period, pHi was restored to that observed at the beginning of the experiment. In contrast, when embryos that had been vitrified were examined immediately following warming, the increase in pHi induced by removing Cl^- from the medium was reduced by half compared to the controls ($P < 0.05$; Table III). The rate of recovery from alkalosis induced by NH_3 was also significantly decreased in vitrified 2-cell embryos examined immediately following warming (Table III). Consequently, the final pHi at the end of the recovery period was also significantly higher in these embryos (Figure 2). Embryos that were examined 2 h after warming exhibited an increase in pHi when Cl^- was removed from the medium. This increase in pHi was similar in magnitude to that in the control embryos. However, the rate of recovery from the induced alkalosis was still significantly slower in these embryos compared to the control embryos (Table III). Therefore, the final pHi at the end of this recovery period was significantly higher than in the control vitrified embryos. A similar pattern of pHi regulation was observed in embryos that were examined 4 h after warming. The increase in pHi induced by Cl^- was similar to the control embryos although the rate of recovery was significantly less (Table III). However, 2-cell embryos 4 h after warming were able to restore pHi to initial values, although the time taken to recover pHi was slower than the control embryos (Figure 2). By 6 h after warming, the pattern of recovery from alkalosis by 2-cell embryos that had been vitrified was equivalent to that of the control embryos (Table III; Figure 2).

Effect of an increase in pHi for 4 h on 2-cell embryo metabolism

Effects on both oxidative and glycolytic activity were assessed after increasing the pHi by 0.097 ± 0.008 ($n = 100$ embryos) or 0.147 ± 0.012 ($n = 100$ embryos) pH units by incubation with either 5 or 10 mmol/l trimethylamine (TMA) respectively. Incubation with 5 mmol/l TMA did not affect glycolytic activity, however incubation with 10 mmol/l TMA for 4 h significantly increased glycolytic activity of fresh 2-cell embryos (Figure 3a). In contrast, incubation with 5 or 10 mmol/l TMA for just 4 h substantially impaired oxidative capacity of 2-cell embryos (Figure 3b).

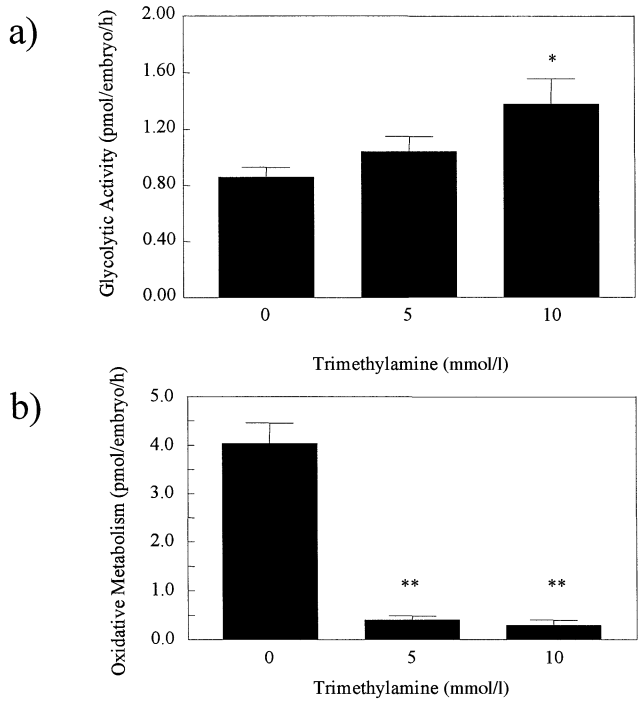

Figure 3 a,b. Effect of increased pHi by incubation with trimethylamine (TMA) for 4 h on embryo metabolism. **(a)** Glycolytic activity (pmol/embryo); **(b)** oxidative metabolism (pmol/embryo). * Significantly different from control embryos ($P < 0.05$); ** significantly different from control embryos ($P < 0.01$).

Discussion

The ability to regulate intracellular homeostasis is essential for normal cellular development. It is evident from this study that the two major transport systems that regulate pHi in the hamster 2-cell embryo are significantly impaired during the cryopreservation procedure used in this study. It remains to be ascertained in ongoing studies which aspect of the cryopreservation procedure (e.g. cooling, vitrification solutions) is responsible for the alterations in the regulation of pHi. Normal activity of both the Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger was not re-established until between 4 and 6 h after warming. This inability to regulate pHi was manifest as both a reduced ability to recover from either an acid or an alkaline challenge and also as an inability to maintain resting pHi at the normal value of 7.2. Instead, pHi was increased by around 0.2 pH units. It has been reported in the mouse (Zhao and Baltz, 1996), hamster (Lane *et al.*, 1999a) and cow (Lane and Bavister, 1999) that an increase in pH induced by a membrane-permeant weak alkali impairs development in culture.

Activities of the Na^+/H^+ antiporter and the HCO_3^-/Cl^- exchanger are important for normal embryo development in culture. When either transporter is inhibited, development is reduced. It appears that the reduction in both Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger activities results in an abnormal increase in the baseline pHi. The mechanisms for the

decrease in activity of the pHi regulatory transport proteins following cryopreservation and warming are unknown. Both the Na^+/H^+ antiporter and the HCO_3^-/Cl^- transporter activities are dependent on their sensitivity in detecting alterations in pHi from the normal set-point (Lane *et al*., 1998; 1999a). For both transporters, increasing the magnitude of the alterations from the set-point increases the activity of the transporters. In vitrified hamster embryos examined 0 or 2 h after warming, it is clear that this sensitivity to the alterations in cytoplasmic pH by the transporters is impaired. In the case of the Na^+/H^+ antiporter, a change in the phosphorylation or stimulation of the antiporter is normally manifest as an alkaline shift in the pH dependency curve (Grinstein and Rothstein, 1986). This was not observed in the present study (data not shown). Rather it appears that there is a desensitization of the activation system for both the Na^+/H^+ antiporter and HCO_3^-/Cl^- transporter systems. Because both of these transport systems operate by a transmembrane gradient, for Na^+ and Cl^- respectively, it is possible that, in cryopreserved embryos immediately after warming, these gradients are disturbed, which reduces the ability to transport either H^+ or HCO_3^-. Although this study focused on the transporters for the regulation of pHi, it is possible that other transporter systems and regulatory channels will be similarly affected following cryopreservation. Activity of the facilitated glucose transporter GLUT 1, which is expressed in mouse embryos, is also significantly impaired in blastocysts that had been cryopreserved at the 2-cell stage (Uechi *et al*., 1997). In addition to membrane pHi regulation, mitochondrial pH and Ca2+ regulatory mechanisms may also be affected.

Intracellular pH is known to be a potent regulator of cell metabolism and energy production (Busa and Nuccitelli, 1984). Therefore, the increase in pHi of around 0.2 pH units that was observed for 4 h following warming may cause aberrations in several cellular functions. Incubating 2-cell embryos for 4 h with the weak alkali TMA increased pHi by around 0.2 pH units, the same as the increase in pHi induced by cryopreservation. Increasing the pHi of these embryos significantly altered embryo metabolism. Glycolytic activity was increased by incubation with 10 mmol/l TMA and oxidative capacity was substantially reduced by both 5 and 10 mmol/l TMA.

The flux-generating step of glycolysis, the enzyme phosphofructokinase (PFK) is very sensitive to changes in pH (Paetkau and Lardy, 1967). Small increases in PFK activity can cause disproportionately large increases in glycolytic pathway activity. In some cells, PFK activity can be altered 10- to 20-fold by a pH change of only around 0.1 units (Danforth, 1965; Trivedi and Danforth, 1966). The increase in pHi that was observed in hamster embryos for 4 h after warming would stimulate the enzyme PFK resulting in increased glycolytic activity. Indeed, bovine blastocysts assessed immediately after cryopreservation had an increased rate of glycolysis compared to the same embryos measured before freezing (Gardner *et al*., 1996). However, the biggest effect of an increase in pHi on hamster embryo metabolism was a reduction in oxidative metabolism. Oxidative metabolism, specifically metabolism of lactate, appears to be the preferred energy-generating pathway of the cleavage stage hamster embryo (McKiernan *et al*., 1991). Interestingly, an increase in glycolysis by cleavage stage hamster embryos resulted in impaired oxidative metabolism (Seshagiri and Bavister, 1991). A similar effect has been linked to the '2-cell block' in mouse embryos (Gardner and Lane, 1993). This phenomenon, which was first described in rapidly dividing tumour cells, is known as the Crabtree effect (Crabtree, 1929; Koobs, 1972).

In addition to the reduced activity of the pHi transport systems Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger, the intrinsic buffering capacity of hamster 2-cell embryos that had been cryopreserved was reduced immediately following warming. It has recently been reported that the addition of Eagle's non-essential amino acids significantly enhances the ability of mouse cleavage stage embryos to buffer both an acid (Edwards *et al*., 1998) and alkaline load (L.J.Edwards and D.K.Gardner, personal communication). Therefore, it may be prudent to include these amino acids in the culture medium for embryos following cryopreservation when the embryo has a reduced ability to regulate pHi. While some of these amino acids were present in the culture medium for the hamster embryo in the present study, their concentration is 10 times lower than that reported to help in the buffering of mouse embryos.

In conclusion, this study demonstrated that 2-cell hamster embryos that were vitrified had an elevated pHi immediately after warming. The activities of the major pHi regulatory systems in the hamster embryo, the Na^+/H^+ antiporter and HCO_3^-/Cl^- exchanger, were significantly reduced after freezing, and were not restored until 6 h after warming. During this time, the embryos have a significantly reduced ability to regulate pHi, which could have major effects on their subsequent developmental competence.

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