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VITRIFICATION OF BIOPSIED MOUSE EMBRYOS

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Solid surface vitrification (SSV) was compared with in-straw vitrification for cryopreservation of biopsied mouse embryos. Eight-cell stage embryos were zona drilled and one blastomere was removed. Developed morulae or blastocysts were vitrified in microdrop (35% EG + 5% PVP + 0.4 M trehalose) or in straw (7.0 M EG + 0.5 M sucrose). Following recovery, embryos were cultivated *in vitro* or transferred into recipients. Cryopreservation had an effect not only on the survival of biopsied embryos but also on their subsequent development *in vitro*. Cryosurvival of biopsied morulae vitrified in straw was significantly inferior to SSV. The post-warm development of biopsied and non-biopsied morulae was delayed on Day 3.5 and 4.5 in both vitrification groups. A delay in development was observed on Day 5.5 among vitrified non-biopsied blastocysts. The percentage of pups born from biopsied morulae or blastocysts following cryopreservation did not differ from that of the control. No significant differences could be detected between methods within and between embryonic stages in terms of birth rate. The birth rate of biopsied embryos vitrified in straw was significantly lower compared to the non-biopsied embryos. The novel cryopreservation protocol of SSV proved to be effective for cryopreservation of morula- and blastocyst-stage biopsied embryos.

Key words: Embryo biopsy, cryopreservation, mouse, solid surface vitrification

Genotyping of embryos for the determination of sex, a desired trait or inherited disorders before transferring to a recipient is recently gaining great importance due to the increased efficiency of molecular biological methods. Both animal husbandry (Bodó et al., 2001) and human medicine (Handyside et al., 1989, 1998) can benefit from the recent advances. In preimplantation genetic diagnosis, either a cell (a blastomere) is biopsied from an early-stage embryo, or a clump of cells is removed from a blastocyst. In the latter case, the zona pellucida is usually removed, reducing mechanical protection for the embryo. Although the diagnosis on the removed cells can be done relatively quickly (in 4–6 h), a more extensive diagnosis requires time and the biopsied material to be transferred to a specialised centre. Sometimes no recipient is readily available. In such cases cryopreservation of the biopsied embryos is necessary.

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The extensive use of mouse embryos for biopsy and cryopreservation studies explains the large amount data gathered compared to other mammals. The biopsy procedure itself has been shown to have no negative effect on the developmental potential of mouse embryos either when the zona was punctured (Wilton and Trounson, 1989; Krzyminska et al., 1990) or drilled (Garrisi et al., 1992; Bodó et al., 2002).

The cryosurvival following warming and subsequent development of a biopsied and cryopreserved embryo depends on several factors:

(1) *Origin of the embryo. In vitro* produced embryos are often more sensitive to cryopreservation. This is probably due to the high lipid content of *in vitro* produced embryos. Elevated lipid level in the cytoplasm results in increased chilling sensitivity that seriously increases cryomortality of the embryo during cryopreservation (Leibo and Loskutoff, 1993; Massip et al., 1995).

(2) *Developmental stage of the embryo.* Eight-cell (Krzyminska et al., 1990; Liu et al., 1993; Snabes et al., 1993) and morula (Nowshari and Brem, 2000) stages have been found to be the most suitable for biopsy and cryopreservation.

(3) *Degree of zona absence.* Zona penetration resulting in a small defect in the zona pellucida (as in subzonal sperm injection) did not significantly affect the rate of blastocyst formation in cryopreserved 2-cell embryo following controlled-rate freezing compared to zona-intact cryopreserved embryos (Lee et al., 1997). However, when a larger portion of the zona was removed (partial zona dissection), blastocyst formation of cryopreserved embryos significantly decreased. It was concluded that embryos with a persistent hole on the zona are less suitable for cryopreservation. These results were contradictory with those of Garrisi et al. (1992) who found that cryopreservation of embryos and even oocytes may succeed without an intact zona pellucida.

(4) *Method of biopsy.* This does not seem to be a factor that significantly affects cryosurvival. The biopsied embryo, when the zona damage resulted from a mechanical puncture, had a similar survival and development (blastocyst formation) after cryopreservation as the zona-intact ones (Liu et al., 1993; Snabes et al., 1993). Krzyminska and O'Neill (1991) found that cryopreservation affected negatively only the survival of biopsied embryos, with no direct interference on development. Zona drilling with acidic Tyrode's solution was also found to have no negative effect on the development of cryopreserved early-stage embryos (Gordon and Gang, 1990; Depypere et al., 1991; Garrisi et al., 1992).

(5) *Proportion of cell number reduction.* Liu et al. (1993) studied the effect of the degree of biopsy (cell loss) on 8-cell mouse embryo development. Biopsy-created zona defects and cell number reduction had no negative consequences on embryo development *in vitro,* when fewer than four blastomeres were removed. *In vivo* development was impaired only when more than two blastomeres were removed. Survival and development *in vitro* and *in vivo* of biopsied embryos was not affected by slow-rate freezing (Liu et al., 1993).

(6) *Rate of cooling and warming.* The method of cryopreservation does not seem to be a factor that affects the survival of biopsied embryos: good results could be achieved either with conventional freezing (Garrisi et al., 1992) or vitrification (Nowshari and Brem, 2000).

The goal of the present study was to compare a new method of vitrification performed in microdrop (solid surface vitrification; SSV; described by Dinnyés et al., 2000) with an 'in-straw' vitrification method (Nowshari and Brem, 1998) in preserving biopsied mouse embryos. Eight-cell stage embryos were biopsied and cryopreserved in morula or blastocyst stage. The effect of biopsy and cryopreservation on the *in vitro* and *in vivo* development of the embryos was evaluated.

Materials and methods

All chemicals were 'cell-culture' or 'embryo tested' and purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Embryo source

Mice were kept in a 12 h/12 h dark/light cycle and fed *ad libitum.* Embryos were obtained from (C57BL/6J × CBA) F_1 females treated with 5 international units (IU) of pregnant mare serum gonadotropin (Folligon, Intervet, Boxmeer, The Netherlands) followed 46 h later by 5 IU human chorionic gonadotropin (Werfachor, Alvetra und Werfft AG, Wien, Austria) and caged with a male overnight. Females with a copulation plug next morning were considered pregnant and were sacrificed by cervical dislocation two days *post coitum (p.c.).* Embryos of 8-cell stage were flushed from the oviducts with M2 medium. Embryos were cultured *in vitro* in 20 µl drops of M16 medium (Hogan et al., 1994) covered with mineral oil (Cat. No.: M-8410) at 37 °C in an atmosphere of 5% CO_2 in air.

Micromanipulation

Eight-cell stage embryos were biopsied as described earlier by Bodó et al. (2002). The embryos were pre-treated in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) at 37 °C for 20 min to loose the tight junctions between the cells. An embryo was placed in a 10-µl droplet of M2 medium covered with mineral oil (Cat. No.: M-8410). The manipulation was performed at 37 °C on an inverted microscope (IMT2, Olympus, Japan) using two sets of Narishige micromanipulators. The embryo was fixed with the vacuum of the holder capillary. With a drilling capillary filled with acidic Tyrode's medium, a hole with a diameter of 25–30 µm was made on the zona. The vacuum of the holder was interrupted and, with the help of the drilling capillary, the embryo was turned with 180° degrees. With a

weak negative pressure generated again in the holder capillary, a blastomere was aspirated through the hole into the holder. The holder with the embryo was moved to the edge of the medium droplet and the oil layer separated the blastomere from the embryo. The 7-cell embryo was submitted to cryopreservation.

Cryopreservation

The micromanipulated embryos were cultivated *in vitro* in M16 medium, as described above, until the morula (2.5 Day *p.c.*) or blastocyst (3.5 Day *p.c.*) stage, when they were cryopreserved using one of the two vitrification protocols, then stored in liquid nitrogen for one to three weeks. After warming and rehydration, they were either cultured further *in vitro* or – when they had reached the blastocyst stage – transferred into recipients.

Solid surface vitrification (SSV). The protocol was adapted from the method described by Dinnyés et al. (2000) for vitrification of bovine oocytes. Embryos were washed three times in Medium 199 (Cat. No.: M4530) supplemented with 20% fetal calf serum (FCS, Cat. No.: 10082147, Invitrogen, USA) and placed into 4% ethylene glycol (EG; Riedel-de Haën, Germany, Cat. No.: 34907) in Medium 199 plus 20% FCS at 37 °C for 15 min. After equilibration, the embryos were transferred to and washed in small drops of vitrification solution (VS) consisting of 35% EG, 5% polyvinyl-pyrrolidone (PVP; molecular weight: 40,000, Cat. No.: P-0930) and 0.4 M trehalose (Cat. No.: T-0167) in Medium 199 and 20% FCS for 30 sec, then loaded with a small amount of solution into a glass capillary. Embryos were expelled with a small volume of medium from the pipette onto a metal surface precooled in liquid nitrogen, where they vitrified instantaneously in microspheres. The spheres were placed into cryovials (Nunc, Denmark) with precooled fine forceps and stored under liquid nitrogen. The embryos were warmed by dropping the spheres with forceps into 2 ml of 0.3 M trehalose in Medium 199 plus 20% FCS at 37 °C for 3 min. Embryos were then washed three times in Medium 199 plus 20% FCS and three times in M16 and transferred to culture.

Vitrification in ethylene glycol. The method is based on that of Nowshari and Brem (1998). First, the embryos were partially dehydrated in 1.5 M EG and 0.25 M sucrose in M2 at room temperature for 5 min. Then 0.25 ml plastic insemination straws (IMV, France) were loaded with three columns of liquids: (1) 170 µl of 0.5 M sucrose (Cat. No.: S-9378), (2) 20 µl vitrification solution consisting of 7.0 M EG and 0.5 M sucrose, and (3) 50 µl of vitrification solution containing the embryos. All solutions were made with M2 supplemented with 10% FCS. Loading was performed with a syringe attached to the plugged end of the straw. Solution columns were separated with air bubbles. The function of the second column was to prevent the sucrose solution from leaking into the third column. The straws were heat sealed and lowered vertically, immediately but slowly into liquid nitrogen. Thawing was performed in a 30 °C water bath. Dilution of the cryoprotectants and rehydration of the embryos was achieved by ex-

pelling the content of the straw into 0.5 M sucrose at room temperature. After 5 min, the embryos were washed three times in M2 and in M16 and transferred to *in vitro* cultivation.

Evaluation of viability of embryos

Blastocyst formation and hatching rate were recorded between Day 3.5 and 5.5 *p.c.* In the blastocyst, recovery of blastocoel and hatching rate were observed between Day 4.5 and 5.5 *p.c.* Some embryos were transferred into pseudopregnant recipients. Under anaesthesia with Avertin, 4–8 embryos were transferred surgically into each horn of the uterus. Pregnancies were allowed to term.

Statistical analysis

Differences between treatment groups were tested for significance with the chi-squared test using INSTAT (GraphPad Software, V2.05a). Differences were considered significant at $P < 0.05$.

Results

Development in vitro

Survival and *in vitro* development of non-biopsied cryopreserved embryos are shown in Table 1. The survival of morulae following vitrification in straw or by SSV did not differ from that of the control. By Day 3.5 *p.c.,* significantly fewer embryos developed into blastocyst in the vitrification groups than in the non-vitrified control group. By Day 4.5 *p.c.,* the rate of hatched blastocyst was significantly lower in the vitrification groups than in the control group. By Day 5.5 *p.c.,* no difference could be detected in the rate of blastocyst formation and hatching between the cryopreserved and the control groups. The survival of blastocyst vitrified in straw or by SSV did not differ from the control. On Day 4.5 *p.c.,* no difference was detected between the vitrified groups and the control group in terms of blastocyst or hatched blastocyst rate. By Day 5.5 *p.c.,* significantly less blastocyst hatched in the cryopreserved group than in the control group.

Survival and *in vitro* development of biopsied and vitrified embryos are shown in Table 2. The cryosurvival of biopsied morulae was not different from the control when vitrified by SSV but significantly reduced when cryopreserved in straw. In both vitrification groups, blastocyst formation by Day 3.5 *p.c.* was inferior compared to the control. A delay in hatching was observed on Day 4.5 *p.c.* among blastocysts developed from vitrified morulae. By Day 5.5 *p.c.,* no difference could be observed in blastocyst and hatched blastocyst rates between the vitrification groups and the control group. No difference was found in survival and in development of biopsied blastocysts between the cryopreservation groups and the control group.

Table 1: Survival and *in vitro* development of non-biopsied, cryopreserved embryos

Stage	Treatment	Vitrified	Survived (%)	Day 3.5		Day 4.5		Day 5.5	
				Total blastocyst (%)[1]	Hatched blastocyst (%)[1]	Total blastocyst (%)[1]	Hatched blastocyst (%)[1]	Total blastocyst (%)[1]	Hatched blastocyst (%)[1]
Morula (Day 2.5)	In straw	40	38 (95)	15 (39)[b]	0	36 (95)	13 (34)[d]	35 (92)	29 (76)
	SSV	36	35 (97)	13 (37)[b]	0	32 (91)	10 (29)[d]	27 (77)	23 (80)
	Control		37	32 (86)[a]	0	35 (95)	23 (62)[c]	32 (86)	28 (84)
Blastocyst (Day 3.5)	In straw	57	52 (91)			52 (100)	4 (8)	52 (100)	20 (38)[d]
	SSV	67	63 (94)			63 (100)	4 (6)	60 (95)	23 (37)[d]
	Control		54			54 (100)	3 (6)	54 (100)	38 (70)[c]

[1]Embryo development is given in percent of surviving embryos; [a, b, c, d]Values with different superscripts within the same column and embryo stage differ significantly (χ^2 $P < 0.05$)

Table 2: Survival and *in vitro* development of biopsied and cryopreserved embryos

Stage	Treatment	Vitrified	Survived (%)	Day 3.5		Day 4.5		Day 5.5	
				Total blastocyst (%)[1]	Hatched blastocyst (%)[1]	Total blastocyst (%)[1]	Hatched blastocyst (%)[1]	Total blastocyst (%)[1]	Hatched blastocyst (%)[1]
Morula (Day 2.5)	In straw	32	24 (75)[a]	8 (33)[a]	1 (4)	24 (100)	11 (46)[a]	24 (100)	22 (92)
	SSV	35	30 (86)[b]	13 (43)[a]	3 (10)	30 (100)	13 (43)[a]	30 (100)	25 (83)
	Control		34[b]	31 (91)[b]	3 (9)	34 (100)	24 (71)[b]	32 (94)	30 (88)
Blastocyst (Day 3.5)	In straw	24	24 (100)			24 (100)	9 (38)	23 (96)	13 (54)
	SSV	29	28 (97)			28 (100)	7 (25)	28 (100)	16 (57)
	Control		27			27 (100)	12 (44)	27 (100)	21 (78)

[1]Embryo development is given in percent of surviving embryos; [a, b]Values with different superscripts within the same column and embryo stage differ significantly (χ^2 $P < 0.05$)

Comparison of the data of Tables 1 and 2 revealed that the cryosurvival of morulae vitrified in straw significantly decreased following biopsy. A higher rate of hatched blastocysts was observed among biopsied blastocysts by Day 4.5 *p.c.,* either vitrified or not.

Development in vivo

In vivo development data of cryopreserved embryos (biopsied and non-biopsied) are shown in Table 3.

Birth rate of embryos developed from transferred non-biopsied morulae vitrified in straw was 77%, not different from the birth rate following the transfer of morulae cryopreserved by SSV (76%) or from the non-cryopreserved control (81%). When blastocysts were cryopreserved and transferred, the birth rates for in-straw vitrification and SSV were 85% and 75%, respectively, not different from the control.

The percentage of pups born from biopsied and cryopreserved morulae was 52% for in-straw vitrification, and 64% for the SSV. In case of blastocysts the respective results were 47% and 53%. Birth rate of transferred control (biopsied, not cryopreserved) embryos was 65%. No significant difference could be detected between methods or between stages in terms of birth rate.

When birth data of vitrified non-biopsied embryos were compared with those of the vitrified biopsied embryos (effect of biopsy), it was found that biopsy significantly reduced the birth rate when embryos were vitrified in straw. When biopsied embryos were cryopreserved by SSV, no significant reduction in birth rate could be detected.

In all experiments, newborn pups were active, apparently healthy, with no visible signs of defects.

Table 3

In vivo development of intact and biopsied, cryopreserved embryos

Stage	Cryo-preser-vation	Intact			Biopsied		
		Transferred	Recipient	Pups (%)[1]	Transferred	Recipient	Pups (%)[1]
Morula	In straw	39	4	30 (77)[a]	42	5	22 (52)[b]
	SSV	21	3	16 (76)	39	4	25 (64)
Blastocyst	In straw	20	2	17 (85)[a]	15	2	7 (47)[b]
	SSV	24	2	18 (75)	17	2	9 (53)
Blastocyst	Control	32	3	26 (81)	40	4	26 (65)

[1]Birth rates are given in percent of transferred embryos; [a, b]Values with different superscripts within the same row differ significantly (χ^2 P < 0.05)

Discussion

Our experiments showed that following vitrification high *in vitro* and *in vivo* development rates can be achieved, which are not different from the non-cryopreserved controls.

In conformity with our findings, Nowshari and Brem (2000) found no difference in the rate of live fetuses in pregnant recipients on Day 15 of pregnancy between those transferred with vitrified (45%) and non-vitrified non-biopsied embryos (51%). Our data on survival of vitrified-warmed non-biopsied morulae was not different from their result (91%). Our data on cryosurvival and birth rate of biopsied and vitrified embryos was comparable to their findings obtained from refreezing biopsied 8-cell embryos (89% and 40%, respectively). Bagis et al. (2002) compared the same protocols (i.e. vitrification in straw and by SSV) in the cryopreservation of non-biopsied pronuclear-stage mouse embryos. In their experiments, SSV resulted in a significantly higher survival (96%) and cleavage (80%) than in-straw vitrification (68% and 66%, respectively), but both methods proved to be inferior compared to the cleavage in control (92%). A possible delay in embryonic development could not be judged as no further *in vitro* cultivation data were reported. After the transfer of two-cell embryos, pregnancy was produced in similar percent, and no difference was found in birth rate between the vitrification groups (21% and 20%) and the control group (27%). Our *in vivo* data comply well with their observation that no significant difference was found between the birth rates of embryos vitrified by different methods.

In our experiments, cryosurvival of embryos vitrified by the SSV method was not different from the control and was superior to in-straw vitrification survival values when biopsied morulae were cryopreserved. By Day 3.5, significantly fewer vitrified morulae developed into blastocysts than controls. This delay could be observed in non-biopsied and biopsied embryos as well. By Day 4.5 *p.c.,* the delay manifested in a significantly lower rate of hatched blastocysts compared to the control. By Day 5.5 *p.c.,* however, no difference could be detected in blastocyst formation or hatching between the vitrification groups and the control group. When non-biopsied blastocysts were vitrified, a delay in development was found only on Day 5.5, indicated by a lower hatching rate. However, no such delay was apparent in the development of biopsied blastocysts. The slower development and lower hatching rates may reflect an effect of cryopreservation on embryo metabolism. The observation of Krzyminska and O'Neill (1991) was not reinforced by our findings: the cryopreservation had an effect not only on the survival of biopsied embryos but also on their subsequent development *in vitro.* The higher rate of hatched blastocysts observed among biopsied embryos on Day 4.5 *p.c.* may be explained by the mechanical effect of biopsy: the embryos were freed from their zonae pellucidae.

No significant differences could be detected between methods and between embryonic stages in terms of birth rate. When the effect of biopsy on cryopreservation was evaluated, it was found that the birth rate of biopsied embryos vitrified in straw was significantly lower compared to that of the non-biopsied embryos. Our results demonstrated that although both vitrification methods could result in live pups from transfer of biopsied and cryopreserved embryos, the use of solid surface vitrification was found to be more effective in terms of live pups.

The novel cryopreservation protocol of SSV proved to be very effective for cryopreservation of morula- and blastocyst-stage biopsied embryos. In terms of *in vivo* development, the contribution of the embryos to progeny did not differ from that of the control. Thus, the method described in this paper will be suitable for practical applications in the future.

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