VITRIFICATION OF MICE EMBRYOS IN DIFFERENT DEVELOPMENTAL STAGES USING FOUR VITRIFICATION METHODS

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ABSTRACT

The aim of our study was to test four vitrification methods for preservation of mice embryos, in different developmental stages, using 6 mol/l glycerol and 20 % sucrose as vitrification media. Morula stage embryos were vitrified with OPS straws or classic straws using two equilibration steps, with a hatching rate of 50 % and 47.62 % respectively. Blastocyst stage embryos survived better in 0.25 ml straws with three equilibration steps, 36.84 % hatching rate. Two-cell embryos are sensitive to freezing, but satisfactory results could be obtained with OPS vitrification, hatching rates of 22.73 %. The success of the vitrification technique depends on the developmental stage of the embryo and the method used.

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Introduction

Cryobiology is an interdisciplinary science which studies the physical and biological behavior of tissues and cells (including interactions with media) at low temperatures (generally much lower than the freezing point of water). The metabolism of living cells is dramatically reduced at low temperatures, a fact that allows long-term preservation of cells and tissue (9).

The main steps of the cryopreservation process, regardless of the technique used, are: adding cryoprotectors to cells or tissue before cooling (equilibration step); cooling the cells at a low temperature at which the cells can be stored (freezing step); thawing the cells and removing the cryoprotectant from the cells (rehydration step). There are many methods for embryo freezing and they can be divided into two main categories: conventional freezing (equilibrated or controlled) and vitrification (unequilibrated or fast). Both are used with success all over the world for freezing oocytes, embryos, sperm and cells.

Mice embryo cryopreservation at -196 °C, without ice crystal formation was reported in 1985 as an alternative to classic freezing methods. Since then the vitrification technique has become more and more a component of Assisted Reproductive Technologies, being an alternative to slow freezing/fast thawing protocols (16).

Vitrification is unequilibrated freezing of the cells, with high concentrations of cryopotectors (4 mol/L to 8 mol/L) and high temperatures decreasing rates from 500 ºC/min to 20 000 ºC/min. If standard, 0.25 mL, straws are used the temperature dropping rates are between 500 ºC/min to 3000 ºC/min, but they can increase to 9000 ºC and 20 000 ºC (13, 21), when live cells are in direct contact with liquid nitrogen, as in Solid Surface Vitrification (SSV) and Cryoloop Vitrification (CLV) (2, 8).

Vitrification can ensure pregnancy rates comparable with classic freezing. Pregnancy rates reported for bovine embryos are 44.5 % for vitrification and 45.1 % for embryos frozen by classic freezing, classic freezing is still used in current embryo transfer technologies because of the fact that the pregnancy rates are satisfactory and there is no significant gain in vitrification (11).

The aim of our study was to test four vitrification methods for preservation of mice embryos, in different developmental stages, using 6 mol/L glycerol and 20 % sucrose as vitrification media.

Materials and Methods

Biological material

The experiments were performed on 27-day-old NMRI mice, females and males, with proven fertility. The animals were subjected to a controlled light regime of 12 h light and 12 h dark, from 9^{00}AM to 21^{00} PM, food and water administered *ad libitum*. The animals' housing and manipulation was in accordance with the international animal welfare legislation.

Female superovulation

The hormones used for superovulatory treatment in mice females are eCG (Equine Chorionic Gonadotropine) and hCG (human Chorionic Gonadotropine). In our experiments we used the following superovulatory scheme: 5 IU (international units) eCG administrated (Folligon, Intervet) (day 0) and 48 h later 5 IU hCG (day 2) (Chorulon, Intervet) was administrated. Following hCG administration the females were mated (female:male ratio 1:1) and the vaginal plug was checked in the morning of the 3rd day of the protocol.

Embryo recovery

Embryos were recovered by perfusion of the oviducts and uterine horns (depending of the stage of the embryo) with recovery media (M2 Sigma M7167). For the embryos at the 2-cell stage and morula stage the oviducts were perfused; for embryos at the blastocyst developmental stage the uterine horns of the females were perfused. After recovery the embryos were morphologically evaluated and only good quality embryos (quality code 1 and 2) were used for vitrification.

Virification in 0.25 ml straws

After recovery the embryos were equilibrated thermally and osmotically at room temperature (22 °C).

For embryo vitrification in 0.25 ml straws, we tested three equilibration methods: one-step, two-step and three-step equilibration, for which we prepared four equilibration media and four rehydration media, as shown in **Table 1**. All media were prepared in phosphate buffered saline (PBS), pH 7.4 (Sigma P3813), and were supplemented with 0.4 % BSA (Bovine Serum Albumin, Sigma A 7906).

In one-step equilibration, one equilibration medium was used with 3 mol/L glycerol (Farmachim, p.a.) and 10 % sucrose (Sigma S7903); embryos were equilibrated for 5 min (22 °C) and then vitrified. After thawing, the straw content was emptied into a Petri dish, the rehydration and vitrification media were mixed, and the embryos were rehydrated for 5 min (37 °C).

Two-step equilibration included two equilibration media: one with 1.5 mol/L glycerol (10 min., 22 °C), and one with vitrification media (40 s, 22 °C). Subsequent to equilibration, the embryos were vitrified. After thawing, the embryos were rehydrated in two steps, using one rehydration media with 1.5 mol/L glycerol and 17 % sucrose, and a second rehydration media, with 17 % sucrose. Embryos were left for 5 min in each rehydration media at 37 °C.

Three-step equilibration: consisted of using three equilibration media: the first one with 1 mol/L glycerol (10 min, 22 °C), the second one with 3 mol/L glycerol (5 min, 22 °C), and the third one was the vitrification media (40 s, 22 °C); then the embryos were vitrified. After thawing, the embryos were placed in rehydration media with 3 mol/L glycerol and 17 % sucrose (5 min, 37 °C), then transferred into a media with 1 mol/L glycerol and 17 % sucrose (5 min, 37 °C) and then into 17 % sucrose media (5 min, 37 °C).

After equilibration the embryos were introduced in straws, as shown in **Fig. 1**, and vitrified by plunging the straw directly into liquid nitrogen (LN) (-196 °C). Thawing the embryos was performed by the air/water method described by Robertson (22).

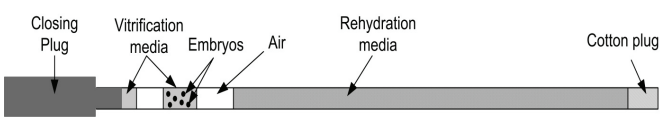

Fig. 1. The method for introducing the embryos in 0.25 mL straws (22).

OPS (Open Pulled Straw) vitrification

Consisted of embryo equilibration in 1.5 mol/L glycerol (10 min, 22 °C), then into vitrification media (1 min, 22 °C), then the embryos were introduced in OPS straws by capillarity, and plugged into LN (-196 °C). Thawing and rehydration was performed by taking the straw out of LN and dipping the tip of the straw directly in a culture media drop. The embryos were expelled in media by covering the end of the straw with the index finger (5 min, 37 °C).

Embryo viability

Viability assessment was performed by culturing the embryos in microdrop culture system, as described by Nagy et. al. (18). Embryo cultivation was performed in a CO_2 incubator, at 37 °C, with 5 % CO_2 in air, atmosphere saturated in water vapors. As culture medium we used KSOM (Potassium Simplex Optimized Medium) supplemented with amino-acids (Sigma M5550) (18).

Statistical analysis

For statistical analysis of the data we used Minitab 15 software. The data were examined for differences by the *chi*-square test, a value of $P < 0.05$ was considered statistically different. In addition, Pearson's contingency coefficient (c) was analyzed for interpretation of the strength of associations.

Results and Discussion

Cryoprotectors are the main component of any cryopreservation media. The penetrating cryoprotectors are used to lower the freezing point of the solution, allowing for longer dehydration. The choice of cryoprotectant is essential: its toxicity must be taken into account first and permeability, second. Moore and Bonilla (17) published a toxicity scale for penetrating cryoprotectors and ethylene glycol was listed as the least toxic one, followed by 1,2-propanol, glycerol, DMSO (Dimethyl sulfoxide), and the most toxic was acetamide. In our vitrification media we used glycerol as the penetrating cryoprotector because it is one of the less toxic and our previous research showed that ethylene glycol has low vitrification abilities when used as a single penetrating cryoprotectant (6).

The results obtained after thawing and culturing the vitrified embryos are presented in **Table 2**.

For the vitrification method with one equilibration step, after thawing and culturing, hatched embryos were obtained only for morula stage embryos (20.83 %, $n = 5$). None of the two-cell and blastocyst stage embryos hatched after cultivation, although the rehydration rate was high (100 % and 86.36 %, respectivey); also the percentage of embryos that resumed development was low (14.29 % for 2-cell embryos and 22.73 % for blastocyst stage embryos). The poor results may be due to an improper thermal or osmotic equilibration of the embryos, or maybe to high concentrations of cryoprotectant used. The addition of nonpenetrating cryoprotector (sucrose) to the equilibration media had no protective effect on the embryos and may be detrimental to embryo viability. Although the cryoprotective mechanism of nonpenetrating cryoprotectors

TABLE 1

Concentrations of cryoprotectors in equilibration and rehydration media

Specification	Glycerol (mol/L)	Sucrose (%)	Usage
Equilibration media	3	10	One-step equilibration
	1.5	-	Two-step equilibration (first medium)
	1	-	Three-step equilibration (first medium)
	3	-	Three-step equilibration (second medium)
Rehydration media	-	17	One-step equilibration Two-step equilibration (second medium) Three-step equilibration (third medium) For loading the straws
	1.5	17	Two-step equilibration (first medium)
	1	17	Three-step equilibration (second medium)
	3	17	Three-step equilibration (first medium)

TABLE 2

Results obtained at thawing the vitrified embryos

Freezing method	Total embryo number	Embryo developmental stage at freezing	Rehydration after thawing		Embryos that resumed development after thawing		Hatching*	
			No.	%	No.	%	No.	%
One equilibration step	21	2 cells	21	100	3	14.29	0	0
	24	Morula	24	100	10	41.67	5	20.83
	22	Blastocyst	19	86.36	5	22.73	0	0.00
Two equilibration steps	23	2 cells	19	82.61	18	78.26	5	21.74^{a}
	21	Morula	21	100	12	57.14	10	47.62^{b}
	22	Blastocyst	15	68.18	5	22.73	2	9.09^{a}
Three equilibration steps	21	2 cells	17	80.95	11	52.38	2	9.52^{aA}
	24	Morula	22	91.67	15	62.50	7	29.17^{a}
	19	Blastocyst	15	78.95	7	36.84	7	36.84^{aB}
OPS vitrification	22	2 cells	22	100	18	81.82	5	22.73^{a}
	20	Morula	20	100	12	60.00	10	50.00^{aA}
	21	Blastocyst	20	95.24	12	57.14	2	9.52^{aB}

* Values with different letters are statistically different ($P < 0.05$, *chi*-square test).

is not fully understood, it seems that they stabilize the membranes, increase the viscosity of the media and allow for lower concentration of penetrating cryprotectors to be used in vitrification media (1, 5, 6, 7, 9, 21, 23), but the addition of nonpenetrating cryoprotectors to equilibration media does not improve the survival rates after thawing (19).

When the vitrification method was with two equilibration steps the highest hatching rate was obtained with morula stage embryos (47.62 %, $n = 12$). The hatching rate of 2-cell embryos was 21.74 % ($n = 5$) and for the blastocyst stage embryos the hatching rate was 9.09 % ($n = 2$). The hatching rate of the embryos at morula stage was significantly higher than the hatching rate of the 2-cell embryos and blastocyst stage embryos when the vitrification technique was with two equilibration steps (χ^2, $P < 0.05$, $c = 0.01$).

Using the vitrification method with tree equilibration steps, we obtained a hatching rate of 36.84 % for embryos at the blastocyst stage ($n = 7$); for embryos at morula stage the hatching rate was 29.17 % ($n = 7$) and for 2-cell embryos the hatching was 9.52 % ($n = 2$). The statistical analysis of the data showed significant differences were observed between the hatching rates of the embryos in blastocyst stage and two cells (χ^2, $P < 0.05$, $c = 0.003$), but no significant difference, between the hatching rate of the embryos at morula and blastocyst stage (χ^2, $P > 0.05$).

When OPS vitrification was used the hatching rate obtained was 22.73 % ($n = 5$) for 2-cell embryos, 50 % ($n = 10$) for

morula stage embryos and 9.52 % (n = 2) for blastocyst stage embryos. At OPS vitrification the hatching rate was significantly higher for morula stage embryos compared with blastocyst stage embryos (χ^2, $P < 0.05$, $c = 0.009$) but there were no differences between the hatching rate of 2-cell and blastocyst stage embryos.

OPS vitrification represented a great step for embryo cryopreservation, the method has the advantage that it uses a small volume of media, assures a better temperature transfer by increasing the temperature decreasing rate, reduces the thermic and osmotic shocks because of the rapid thawing (3, 4, 12, 14, 20, 24).

For the 2-cell embryos hatched blastocyst were obtained at OPS vitrification, at two-step vitrification and at three-step vitrification. The statistical analysis of the data showed no significant differences between the hatching rates at two-step vitrification, three-step vitrification and OPS (χ^2, $P > 0.05$). The results are similar with those in the specialized literature; e.g. Zhang (26) reported that vitrification had an adverse effect on the development of 2-cell embryos.

For the embryos at morula stage the hatching rate was similar for OPS vitrification and two-step equilibration and lower, when one equilibration step and three equilibration steps were used. The statistical analysis showed significant differences only between the hatching rate at OPS and one equilibration step (χ^2, $P < 0.05$, $c = 0.003$). OPS vitrification appears to assure higher hatching rates for embryos in morula stage, compared with 0.25 ml straws probably due to the high rates of temperature drop ensured by the OPS method, as shown by the experiments performed by Gayar and Holtz (10), who concluded that OPS vitrification is better for goat embryos compared with classic straw vitrification.

For blastocyst stage embryos, the highest hatching rate was obtained with three equilibration steps. The hatching rates were lower with OPS and two-step equilibration. There were no hatched blastocysts obtained with the one-step equilibration method. The statistical analysis of the data showed that there were significant differences between the hatching rate of the embryos vitrified with three equilibration steps and two equilibration steps (χ^2, $P < 0.05$, $c = 0.003$), and no significant differences between the hatching rate after two equilibration steps and OPS (χ^2, $P > 0.05$).

The poor results obtained with OPS vitrification and one equilibration step for embryos in the blastocyst stage may be due to the fact that blastocyst stage embryos have a high amount of liquid in their blastocoelic cavity and the equilibration step may have been two short for proper dehydration. Higher survival rates can be obtained for blastocyst stage embryos if artificial reduction of blastocelic cavity is performed before vitrification (25).

Considering the low value of the contingency coefficient (c) the variables observed are independent, suggesting that the vitrification media is not the one responsible for the differences observed, but rather the technique or the developmental stage of the vitrified embryos.

The analysis of the data in **Table 2** reveals that the rehydration rate is not an accurate method for evaluating the viability of embryos after thawing. In the majority of the cases over 90 % of the embryos regained the initial form but did not resume the development after culturing (**Table 2**), and we recommend that resuming the development or hatching rate is more accurate for assessment of the survival of embryos cryopreserved by vitrification.

Conclusions

On the basis of our findings we can conclude that the success of vitrification depends on developmental stage of the embryo. Morula stage embryos can be successfully vitrified with OPS straws or classic straws using two equilibrations steps, blastocyst stage embryos survive better in 0.25 ml straws with three equilibration steps. Two-cell embryos are sensitive to freezing but satisfactory results can be obtained with OPS vitrification.

Our result showed that addition of nonpenetrating cryoprotectors to equilibration media composition do not improve hatching rates post thawing. Rehydration rate of the embryos, post thawing, is not a reliable indicator for the future development of the embryos.

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REFERENCES

1. **Ali J., Shelton J.** (1993) J. Reprod. Fertil., **99**, 471-477.
2. **Bagis H., Sagirkaya H., Mercan H.O., Dinnyes A.** (2004) Molec. Reprod. Dev., **67**(2), 186-192.
3. **Berthelot F., Martinat-Botté F., Perreau C., Terqui M.** (2001) Reprod. Nutr. Dev., **41**, 267-272.
4. **Berthelot F., Martinat-Botte F., Vajta G. , Terqui M.** (2003) Livestock Prod. Sci., **83**, 73-83.
5. **Cean A., Păcală N., Boleman A.** (2009) Scientific Papers: Animal Science and Biotechologies, **42**(1), 14-17.
6. **Cean A., Păcală N., Ivan A., Carabă V., Korbuly B.** (2008) Scientific Papers: Animal Science and Biotechologies, **41**(1), 162-168.
7. **Cean A., Păcală N., Ivan A., Carabă V., Korbuly B.** (2007) Scientific Papers: Animal Science and Biotechologies, **40**(1), 201-206.
8. **Cuello C., Gil M.A., Parrilla I., Tornel J., Vazqueza J.M., Roca J., Berthelot F., Martinat-Botte F., Martinez E.A.** (2004) Theriogenology, **62**, 353-361.
9. **Gao D., Critser J.K.** (2000) ILAR Journal, **41**(4), 187-196.

10. **Gayar M.E.L., Holtz W.** (2001) J. Anim. Sci., **79**, 2436-2438.
11. **Guignot F.** (2005) INRA Prod. Anim., **18**, 27-35.
12. **Kong I.K., Lee S.I., Cho S.G, Cho S.K., Park C.S.** (2000) Theriogenology, **53**(9), 1817-1826.
13. **Leibo S.P., Songsasen N.** (2002) Theriogenology, **57**, 303-326.
14. **Lewis I.M., Lane M.W., Vajta G.** (1999) Theriogenology, **51**, 168.
15. **Liebermann J., Dietl J., Vanderzwalmen P., Tucker M.J.** (2003) Reprod. Biomed. Online, **7**, 623-633.
16. **Liebermann J., Nawrot F., Isachenko V., Evgenia Isacenko, Rahimi G., Tucker M.J.** (2002) Biol. Reprod., **67**, 1671-1680.
17. **Moore K., Bonilla A.Q.** (2006) ARBS Annu. Rev. Biomed. Sci., **8**, 19-32.
18. **Nagy A., Getsenstein M., Vintersten K., Behringer R.** (2003) Manipulating the Mouse Embryo: A Laboratoy Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, New York, pp. 161-189.
19. **Newton H., Fisher J., Arnold J.R., Pegg D.E., Faddy M.J., Gosden R.G.** (1998) Hum Reprod., **13**(2), 376-380.
20. **Nowshari M.A., Brem G.** (2001) Hum. Reprod., **16**(11), 2368-2373.
21. **Rall W.F., Fahy G.M.** (1985) Nature, **313**, 573-575.
22. **Robertson E.D.** (2003) Non Surgical Embryo Transfer, 9th Ed., Harrograde Genetics International, Inc., Tennessee, USA, p.10.
23. **Takahashi T., Hirsh A., Erbe E., Williams R.J.** (1988) Biophys. J., **54**(3), 509-518.
24. **Vajta G., Booth P.J., Holm P., Greve T., Callesen H.** (1997) Cryo-Letters, **17**, 191-195.
25. **Vanderzwalmen P., Bertin G., Debauche Ch., Standaert V., Van Roosendaal E.** (2002) Hum. Reprod., **17**(3), 744-751.
26. **Zhang J., Cui J., Ling X., Li X., Peng Y., Guo X., Heng B.C., Tong G.Q.** (2009) J. Assist. Reprod. Genet., **26**(11-12), 621-628.