Fast Cryopreservation of the Mammalian Embryos in Different Developmental Stages by 0.25 mL Straws Vitrification with One Equilibration Step

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Abstract

The aim of our study was to test the cryoprotective proprieties of 7 vitrification media, designed in our laboratory, using the 0.25 mL straws vitrification method, with one equilibration step. As biological material we used mouse females, age 2 months superovulated with 5UI PMSG (Pregnant Mare Serum Gonadotropine) and 5 UI hCG (human Corionic Gonadotropine). For freezing we used embryos in three developmental stages: 2 cells, morula and blastocyst. After recovery, the embryos were placed in equilibration media, after 5 minutes, the embryos were introduced in straws, in vitrification media and plugged directly into liquid nitrogen. After vitrification the straws were thawed in water bath at 37°C, the embryos were rehydrated for 5 minutes and then *in vitro* cultured. The percent of embryos that rehydrated, resumed development and hatched were registered. The best results were obtained with embryos in morula stage that had a hatching rate of 20.83% when MV1 was used for vitrification. None of the embryos in 2 cells and blastocyst stage hatched after thawing and *in vitro* culture, regardless of the vitrification media used. From the vitrification media tested, the worst results were obtained with MV4 and MV6, none of the embryos reached hatching stage, regardless of the development stage. The vitrification method in 0.25 mL straws, with one equilibration step can be used for cryopreservation of the morula stage embryos, but is ineffective for vitrification of the 2 cells and blastocyst stage embryo. Media VM4 and VM6 are not suited for vitrification in 0.25 mL straws, with one equilibration step, of mouse embryos.

Keywords: mouse embryos, vitrification media, hatching rate, cryoprotectors

1. Introduction

Cryobiology is a multidisciplinary science that studies the physical and biological behaviors of the tissues and cells at low temperatures. Cellular metabolism of living cells decreases dramatically at low temperatures, allowing long-term preservation of cells.

Embryo cryopreservation at -196° C, without ice crystal formation (vitrification) has been mentioned since 1985 as an alternative to traditional (controlled) freezing. Since then, vitrification technique has become increasingly used in assisted reproductive technologies in animals as an alternative to traditional protocols slow freezing / rapid thawing [1].

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Vitrification success lies in freezing medium composition. Vitrification medium must satisfy several conditions: crioprotector concentration must be sufficiently high to prevent formation of ice crystals; the crioprotector used must have low toxicity, so the embryos can tolerate [2].

The aim of our study was to test the cryoprotective proprieties of 7 vitrification media, designed in our laboratory, using the 0.25 mL straws vitrification method, with one equilibration step.

2. Materials and methods

As biological material we used mouse females, age 2 months superovulated with 5UI PMSG (Pregnant Mare Serum Gonadotropine) and 5 UI hCG (human Corionic Gonadotropine). The animals were maintained in 12 hours light and 12 hours dark period and the hormone administration was performed at the middle of the light period.

After the superovulation the females were placed with males and the vaginal plug was verified in the morning of the next day. Only the females with vaginal plug were used for embryo recovery. After recovery embryos were morphological evaluated and only the embryos in quality code 1 and 2 were used for freezing. Embryos in three developmental stages were used for freezing: 2 cells, morula and blastocyst, recovered at different time interval from the vaginal plug discovery.

The vitrification of the embryos in 0.25mL straws, with one equilibration step, was performed as follows: after recovery, the embryos were placed in equilibration media(EM) with ½ **VM** concentration of cyoprotectors (table 1), after 5 minutes the embryos were placed in to VM and loaded in to 0.25mL straws and plunged directly in liquid nitrogen (-196°C).

For freezing we used 7 vitrification media: **VM 1** (6 mol. L-1 glycerol with 20% sucrose), **VM2** (6 mol. L-1 glycerol with 20% Ficoll 70), **VM3** (6.5 mol. L-1 glycerol with 20% galactose), **VM4** (6 mol. L-1 ethylene glycol with 20% galactose), **VM5** (6,5 mol. L-1 DMSO with 15% sucrose), **VM6** (6 mol. L-1 DMSO with 15% Ficoll 70) and **VM7** (5.5 mol. L-1 DMSO with 20% galactose). The vitrification media were designed in our laboratory and resulted from previous research.

The thawing of the embryos was performed as follows: the straws were removed from liquid nitrogen and hold in air for 5 seconds; the straws were placed into water bath at 37°C. After thawing the straws were cut and the embryos were thawed and placed into rehydration media (**RM**). The composition of equilibration media (**EM**) and rehydration media (**RM**) are presented in table 1.

Table 1. The composition of the equilibration and rehydration media used for vitrification of the embryo in 0.25 mL straws, with one-step equilibration

Crt No	Vitrification media (MV)	**Equilibration media (EM)**				**Rehydration media (RM)**	
		Penetrating cryoprotector (M)		Nonpenetrating cryoprotector (%)			
		Specification	CM (moli L^{-1})	Specification	C %	Specification	C %
1	MV 1	Glycerol	3	Sucrose	10	Sucrose	17
2	MV 2	Glycerol	3	Ficoll 70	10	Ficoll 70	17
3	MV 3	Glycerol	3.25	Galactose	10	Galactose	17
4	MV 4	Ethylene glycol	3	Galactose	10	Galactose	17
5	MV 5	DMSO	3.25	Sucrose	7.5	Sucrose	17
6	MV 6	DMSO	3	Ficoll 70	7.5	Ficoll 70	17
7	MV 7	DMSO	2.75	Galactose	10	Galactose	17

After thawing and rehydration the embryos were transferred into culture media (M16) and placed into incubator and their development was monitored. The rehydration rate, the resuming of the development and hatching rate was monitored. The data obtained were statistically analyzed using chi-square test.

3. Results and discussion

The results obtained after *in vitro* culturing the embryos in 2 cell developmental stage, vitrified in 0.25 mL straws, with one equilibration step are presented in table 2.

Table 2. Results obtained after *in vitro* culturing the 2 cell embryo after thawing

Vitrification media	Embryos (No.)	Rehydration after thawing		Embryos that resumed development	
		n	%	n	%
6 mol. L^{-1} glycerol with 20% sucrose (**VM1**)	21	21	100	3	14.29 ^{a}
6 mol. L^{-1} glycerol with 20% Ficoll 70 (**VM2**)	20	18	90	3	15.00 ^{b}
6.5 mol. L^{-1} glycerol with 20% galactose (**VM3**)	21	21	100	16	76.19 ^{a,b,c}
6 mol. L^{-1} ethylene glycol with 20% galactose (**VM4**)	22	22	100	2	9.09 ^{c,d}
6,5 mol. L^{-1} DMSO with 15% sucrose (**VM5**)	22	22	100	7	31.82 ^{c,e}
6 mol. L^{-1} DMSO with 15% Ficoll 70 (**VM6**)	23	22	96	17	73.91 ^{a,b,d,e,f}
5.5 mol. L^{-1} DMSO with 20% galactose (**VM7**)	26	24	92	9	34.62 ^{c,d,f,g}

Values with the same indices are statistically different (chi-square test, $p<0.05$)

From the data presented in table 2 it can be seen that for embryos vitrified with **VM1, VM3, VM4** and **VM5** the rehydration rate was 100%. For the embryos vitrified with **VM6** the rehydration rate was 96% and for embryos vitrified with **VM7** the rehydration was 92%. The lowest rehydration rate was registered for the embryos vitrified with **VM2**, 90% of the embryos regained the initial form.

In respect to the results obtained at culturing the embryos after thawing, the worst results were obtained with **VM4**, only 2 (9.09%) from the 22 embryos vitrified resumed the development. From the 21 embryos vitrified with **VM1** only 3 (14.29%) resumed development. From the 20 embryos vitrified with **VM2** only 3 resumed development (15%). From the 22 embryos vitrified with **VM5,** 7 resumed the development, which represent 31.82 %. From the 26 embryos vitrified with **VM7** 9 resumed the development, which represents 34.62%. From the 23 embryos vitrified with **VM6** 17 resumed development, which represents 73.91%.

The best results obtained at culturing the 2 cells embryos, after vitrification in 0.25mL, with one equilibration step, were obtained for the embryos vitrified with **VM3,** from the 21 embryos vitrified 16 resumed development, which represent 76.19%.

When the data were statistically analyzed there was no significant difference between the differences observed for the embryos that resumed development, after vitrification with **VM1, VM2** and **VM4** ($p>0.05$). The differences observed for the embryos that resumed development after vitrification with **VM3** and **VM6** were not significantly statistically ($p>0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM3** and the percent of embryos that resumed development after vitrification with **VM1, VM2, VM4, VM5, VM7** were statistically significant ($p<0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM6** and **VM1, VM2, VM4, VM5, VM7** were statistically significant ($p<0.05$). Also significant differences were observed between the percent of embryos that resumed development after vitrification with **VM4** and **VM5** ($p<0.05$).

None of the embryos in 2 cell stage reached hatched blastocyst stage, regardless of the vitrificaton media used.

The results obtained after *in vitro* culturing the embryos in morula developmental stage, vitrified in 0.25 mL straws, with one equilibration step are presented in table 3.

Table 3. Results obtained after *in vitro* culturing morula stage embryo after thawing

Vitrification media	Embryos (No.)	Rehydration after thawing		Embryos that resumed development		Hatching	
		no.	%	no.	%	no.	%
6 mol. L^{-1} glycerol with 20% sucrose (**VM1**)	24	24	100	10	41.67 [a]	5	20,83[a]
6 mol. L^{-1} glycerol with 20% Ficoll 70 (**VM2**)	36	34	94.44	5	13.89[a,b]	5	13,89 [b]
6.5 mol. L^{-1} glycerol with 20% galactose (**VM3**)	20	20	100	6	30.0 [c,b]	3	15,00 [c]
6 mol. L^{-1} ethylene glycol with 20% galactose (**VM4**)	26	26	100	4	15.38[a,d]	0	0
6,5 mol. L^{-1} DMSO with 15% sucrose (**VM5**)	21	21	100	13	61.90[b,c,d,e]	2	9,52 [d]
6 mol. L^{-1} DMSO with 15% Ficoll 70 (**VM6**)	23	23	100	8	34,78[f]	0	0
5.5 mol. L^{-1} DMSO with 20% galactose (**VM7**)	24	22	92	6	25.0[e,g]	2	8,33[e]

Values with the same indices are statistically different (chi-square test, p<0,005)

From the data presented in table 3 it can be seen that for embryos vitrified with **VM1, VM3, VM4, VM5** and **VM 6** the rehydration rate was 100%. For the embryos vitrified with **VM7** the rehydration rate was 92% and for embryos vitrified with **VM3** the rehydration was 94.44%.

In respect to the results obtained at culturing the embryos after thawing, the worst results were obtained with **VM2**, only 5 (13.89%) from the 36 embryos vitrified resumed the development. From the 24 embryos vitrified with **VM1** 10 (41.67%) resumed development. Form the 20 embryos vitrified with **VM3** 6 (30.0%) resumed development. From the 26 embryos vitrified with **VM4,** 4 resumed the development, which represent 15.38 %. From the 23 embryos vitrified with **VM6** 8 resumed the development, which represents 34.78%. From the 24 embryos vitrified with **VM7** 6 resumed development, which represents 25.0%.

The best results obtained at culturing the morula stage embryos, after vitrification in 0.25mL, with one equilibration step, were obtained for the embryos vitrified with **VM5,** from the 21 embryos vitrified 13 resumed development, which represent 61.9%.

When the data were statistically analyzed there was no significant difference between the differences observed for the embryos that resumed development, after vitrification with MV1 compared with MV3, VM5, VM6 and VM7 ($p>0.05$). Also there were no significant differences between the differences observed for the embryos that resumed development after vitrification with **VM2** and **VM4** ($p>0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM1** compared with **VM2** and **VM4** were statistically significant ($p<0.05$). Significant differences were observed between the percent of embryos that resumed development after vitrification with **VM2** and **VM3** ($p<0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM5** compared with embryos that resumed development after vitrification with **VM2, VM3, VM4** and **MV7** were statistically significant ($p<0.05$).

In respect to the hatching rate of the embryos none of the embryos vitrified with **VM4** and **VM6** reached this stage. For the embryos vitrified with **VM1** the hatching rate was 20.83% (n=5), for embryos vitrified with **VM2** hatching rate was 13.89% (n=5). The hatching rate of the embryos vitrified with **VM3** was 15.0% (n=3). From the 21 embryos vitrified with **VM5** only 2 hatched (9.52%). The lower rate of hatching was observed for the embryos vitrified with **VM7**, only 2 of the 24 embryos vitrified reached hatched embryo stage, which represent 8.33%.

When the data were statistically analyzed there were no significant differences observed between the hatching rate of the embryos cultured after vitrification regardless of the media used ($p>0.05$).

The results obtained after *in vitro* culturing the embryos in blastocyst, vitrified in 0.25 mL straws, with one equilibration step are presented in table 4.

Table 4. Results obtained after *in vitro* culturing blastocyst stage embryo after thawing

Vitrification media	Embryos (No.)	Rehydration after thawing		Embryos that resumed development	
		no.	%	no.	%
6 mol. L^{-1} glycerol with 20% sucrose (**VM1**)	22	19	86.36	5	22.73[a]
6 mol. L^{-1} glycerol with 20% Ficoll 70 (**VM2**)	26	19	73.08	7	26.92[b]
6.5 mol. L^{-1} glycerol with 20% galactose (**VM3**)	19	13	68	2	10.53[c]
6 mol. L^{-1} ethylene glycol with 20% galactose (**VM4**)	24	20	83	5	20.83[d]
6.5 mol. L^{-1} DMSO with 15% sucrose (**VM5**)	34	26	76	24	70.59[a,b,c,d,e]
6 mol. L^{-1} DMSO with 15% Ficoll 70 (**VM6**)	22	21	95	18	81.82[a,b,c,d,f]
5.5 mol. L^{-1} DMSO with 20% galactose (**VM7**)	22	16	72.73	14	63.64[a,b,c,d,g]

Values with the same indices are statistically different (chi-square test, $p<0,005$)

From the data presented in table 4 it can be seen that for embryos in blastocyst stage vitrified with **VM1** the rehydration rate was 86.36% (n=19). From the 26 embryos vitrified with **VM2** 19 (73.08%) of the embryos regained the initial form. From the 19 embryos vitrified with **VM3** 13 (68%) embryos regained the normal appearance. For the embryos vitrified with **VM4** the rehydration rate was 83% (n=20). From the 34 embryos vitrified with **VM5** 26 (76%) regained the initial form after thawing. For the embryos vitrified with **VM6** the rehydration rate was 95% (n=21) and for the embryos vitrified with **VM7** the rehydration was 72.77% (n=16).

In respect to the results obtained at culturing the embryos after thawing, the worst results were obtained with **VM3**, only 2 (10.53%) from the 19 embryos vitrified resumed *in vitro* development. From the 22 embryos vitrified with **VM1** 5 (22.73%) resumed *in vitro* development. From the 26 embryos vitrified with **VM2** 7 embryos (26.92%) resumed the development. From the 24 embryos vitrified with **VM4,** 5 resumed the development, which represent 20.83 %. From the 34 embryos vitrified with **MV5** 24 resumed *in vitro* development after thawing, which represents 70.59 %. From the 22 embryos vitrified with **MV7** 14 resumed in vitro development, which represents 63.64%.

The best results obtained at culturing the blastocyst stage embryos, after vitrification in 0.25mL, with one equilibration step, were obtained for the embryos vitrified with **VM6,** from the 22 embryos vitrified 18 resumed development, which represent 81.82%.

When the data were statistically analyzed there was no significant difference between the differences observed for the embryos that resumed development, after vitrification with MV1 compared with **VM2, VM3**, and **VM4** ($p>0.05$). Also there were no significant differences between the differences observed for the embryos that resumed development after vitrification with **VM5, VM6** and **VM7** ($p>0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM1** compared with **VM5**, **VM6** and **VM7** were statistically significant ($p<0.05$). Significant differences were observed between the percent of embryos that resumed development after vitrification with **VM2** compared with **VM5, VM6** and **VM7** ($p<0.05$). The differences observed between the percent of embryos that resumed development after vitrification with **VM3** compared with embryos that resumed development after vitrification with **VM5, VM6** and **VM7** were statistically significant ($p<0.05$). Also significant differences were observed between the percent of the embryos that resumed development after vitrification with **VM4** compared with **VM5, VM6** and **VM7** ($p<0.05$).

None of the embryos vitrified in blastocyst developmental stage reached hatched blastocyst stage, regardless of the vitrification media used.

From all the developmental stages tested the best results were obtained with embryos in morula stage. These findings are consistent with speciality literature. A study performed by Guang-Bin Zhou et. al. (2005) showed that all the vitrified-warmed morula had similar blastocyst rate compared to that of control [3]. Also, for rat embryos a study performed by Han M.S. et. al. (2003), showed that rat embryos at the 2-cell to blastocyst stages can be vitrified with EFS40, but morula stage is the most feasible stage for embryo cryopreservation in this species [4].

Our study demonstrates that the morula developmental stage is the most feasible stage for mouse embryo fast cryopreservation under our experimental conditions (0.25 mL straws, with one equilibration step).

4. Conclusions

1. The rehydration rate of the embryos in 2 cell developmental stage, vitrified in 0.25 mL straws, with one equilibration step was contained between 90% (**VM2**, *glycerol 6 mol. L^{-1}, Ficoll 70* 20%) and 100% (**VM1**, *glycerol 6 mol. L^{-1}sucrose 20%*; **VM3** *glycerol 6,5 mol. L^{-1}, galactose 20%*; **VM4** *ethylene glycol 6 mol. L^{-1}galactose 20%*; **VM5**, *dimetilsulfoxide 6,5 mol. L^{-1}, sucrose 15%*);
2. The rehydration rate of the embryos in morula developmental stage, vitrified in 0.25 mL straws, with one equilibration step was contained between 92% (**VM7***, dimetilsulfoxid 5,5 M galactose 20%*) and 100% (**VM1**, *glycerol 6 mol. L^{-1}sucrose 20%*; **VM3**, *glycerol 6,5 mol. L^{-1}, galactose 20%*; **VM4** *etilenglicol 6 mol. L^{-1} galactoză 20%*; **VM5** *dimetilsulfoxid 6,5 mol. L^{-1}, sucrose 15%*; **VM6** *dimetilsulfoxide 6 mol. L^{-1}Ficoll 70 15%*);
3. The rehydration rate of the embryos in blastocyst developmental stage, vitrified in 0.25 mL straws, with one equilibration step was contained 68% (**VM3**, *glycerol 6,5 M, galactose 20%*) and 86.36 % (**VM1**, *glycerol 6 M sucrose 20%*);
4. After vitrification in 0.25 mL straws, with one equilibration step of the embryos in 2 cells, the percent of embryos that resumed development was contained between 9.09% (**VM4**, *6 M ethylene glycol with 20% galactose*) and 73.91% (**VM6**, *6 M DMSO with 15% Ficoll 70*).
5. After vitrification in 0.25 mL straws with one equilibration step of the embryos in morula stage the percent of embryos that resumed development was contained between 13.89 (**VM2**, *6 M glicerol with 20% Ficoll 70*) and 61.90 (**VM5**, *6.5 M DMSO with 15% sucroze*).
6. After vitrification in 0.25 mL straws with one equilibration step of the embryos in blastocyst stage with one equilibration step, the percent of embryos that resumed development was comprise between 10.53 % (**VM3**, *6 M glycerol cu 20% galactose*) and 81.82% (**VM6**, *6 M DMSO with 20% Ficoll 70*).
7. Embryos in two cells stage and blastocyst developmental stage have not reached hatched blastocyst stage after vitrification in 0.25 mL straws, with one equilibration step, regardless of the media used for vitrification.

8. The best at vitrification in 0.25 mL straws with one equilibration step were obtained with embryos in morula stage at which the hatching percent was contained between 9.52% (**MV4,** *6 M ethylene glycol with 20% galactose)* and 20.83% (**MV1** *6M glycerol and 20% sucrose*).

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