Bulletin USAMV-CN, 64/2007 (1-2).

RESEARCH REGARDING CRYOPRESERVATION OF MOUSE MORULAE THROUGH DIFFERENT METHODS

Ciupe Simona, I. Groza, Brînduşa Stegeran, Emoke Pall, Ghe. Ştețca

Universitatea de Ştiințe Agricole şi Medicină Veterinară, Facultatea de Medicină Veterinară, Str. Mănăştur nr. 3-5, simona_ciupe@yahoo.com

Key words: cryopreservation, slow-freezing, quick-freezing, vitrification, morulae

Abstract. "In vitro" development of mouse morulae after cryopreservation through different methods was examined. The slow freezing involved an equilibration in 1,5M ethylene glycol (EG) and cooled at 0,5; 0,7; 1,0 or 1,2^{O}C/min. The quick-freezing involved an equilibration in 3M EG + 0,3M sucrose for 5 minute and 2 minute in nitrogen vapor. The vitrification involved a 3 minute equilibration in 20% EG and 60 seconds in solution containing 40% EG, 18% Ficoll and 10,26% sucrose. In all three methods, the straws were thawed in air for 10 seconds and in water at 25^{O}C for 20 seconds and the embryos culture "in vitro" for 72 hours to estimate blastocyst rate. The "in vitro" development rate with 0,5; 0,7; 1,0 and 1,2^{O}C/min. were respectively 72,7; 72,7; 76,5 and 84,8%.

The "in vitro" survival rates of vitrification and quick freezing (85,7 and 80%) were higher then slow freezing.

INTRODUCTION

The cryopreservation of mammal's oocytes, zygotes, early cleavage stage embryos and blastocysts has become an integral part of most "in vitro" fertilization (IVF) program (6,7).

IVF centers have been using traditional slow-rate or equilibrium freezing protocols successfully. Freezing includes the precipitation of water as ice, with the resulting separation of the water from the dissolved substances. Both intracellular ice crystal formation and the high concentration of dissolved substance pose problems. Therefore, a slow rate of cooling attempts to maintain a very delicate balance between those factors that may result in damage, mostly by ice crystallization but also by osmotic and chilling injury, zona and blastomere (1).

Many studies have been undertaken to reduce the time of the freezing procedure and to try to eliminate the cost of expensive, programmable freezing equipment. One way to avoid ice crystallization damage is using vitrification protocols.

As early as 1978, Leibo S.P., ice crystal-free cryopreservation of mouse embryos at –196^{O}C by vitrification was initially reported in an attempted alternative approach to cryo storage. Approximately 8 years later, the successful vitrification of mouse embryos was demonstrated (2, 4).

Vitrification as an ultra-rapid cooling technique is base on direct contact between the vitrification solution containing the cryoprotectant agents and the liquid nitrogen (5). The protocols for vitrification are very simple and they allow cells and tissue to be place directly into the cryoprotectant and then plunged directly into LN_2 (3).

MATERIAL AND METHOD

Biological materials

The embryos were obtained from C57BL/6 and Swiss Albino mouse line. Female mice were superovulated with 5 UI of pregnant mare serum gonadotrophin (PMSG). The ovulation was induced 48 h later by 5 UI of human serum choriogonadotrophin (HCG).

The successfulness of mating with males was veriffied by the presence of the vaginal plug. Morulae embryos were obtained at 2 – 2,5 days after HCG administration by flushing the uterus using Dulbecco's phosphate buffered saline. Selected morulae were simultaneously frozen by slow-rate freezing, quick-freezing and vitrification. Several days later, the embryos were thawed and cultures in the same manner as fresh embryos (fig. 1).

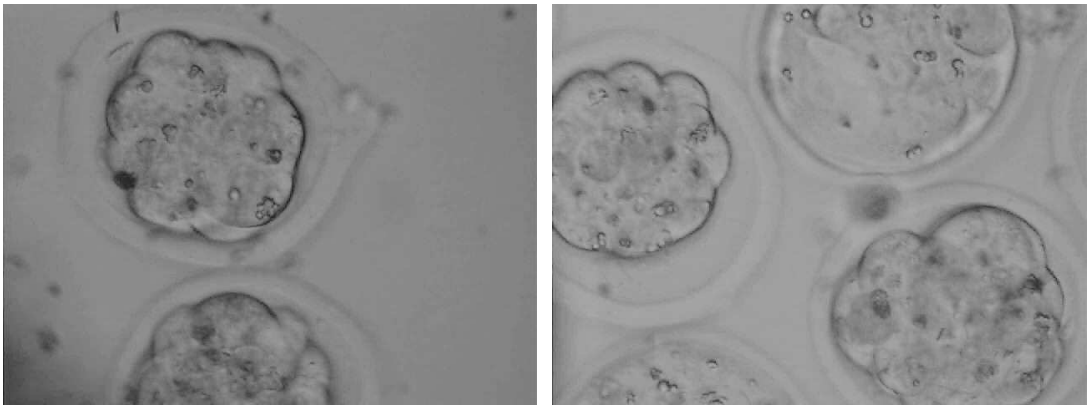


Fig. 1 – Selected morulae for freezing

Freezing procedures

Slow freezing. The compact morulae was equilibrated in 200µl droplets of medium composed of DPBS + 04% BSA + 1,5M ethylene glycol at room temperature for 10 minutes. Embryos were loaded into the 0,25ml straws using two columns of DPBS separated by air bubbles from a central column containing the embryos.

After equilibration, the straws were placed horizontally into the programmable freezer. The straws were cooled at 0,5; 0,7; 1; 1,2^{O}C/min after manual seeding who was carried at – 7^{O}C. when temperature reached – 35^{O}C, the straws were held for 10 minutes and then plunged and stored in liquid nitrogen at -196^{O}C.

Quick freezing. The morulae were equilibrated in 200µl droplets of 3M EG + 0,3M sucrose diluted in DPBS supplemented with 0,4 BSA for 5 minutes at room temperature. Embryos were loaded into a 0,25 ml straws for slow-freezing procedures. After equilibration, the straws were frozen by placing them horizontally in the vapor phase of a nitrogen bath at approximate -170^{O}C. After 2 minutes, the straws were plunged into liquid nitrogen.

Vitrification. The vitrification solution is consisted of 40% EG, 18% Ficoll and 10,26% sucrose diluted in DPBS supplemented with 0,4% BSA. Before vitrification, the morulae were equilibrated in 200µl droplets of 20% EG in DPBS for 3 minutes at room temperature. After this, the embryos were transferred to 200µl droplets of EGS solution for 60 seconds. Embryos were loaded into a 0,25 ml straws as in the slow freezing procedures. For vitrification, the first part of straw filled with DPBS was slowlly immersed into liquid nitrogen, the remaining part of the straw was plunged quickly.

Thawing of frozen embryos. The embryos from freezing methods were thawed by gently agitation for 10 seconds in air and 20 seconds in a 25^{O}C water bath. Immediately after thawing, embryos were expelled and diluted in Petri dishes using the liquid contents of each

straw. After 5 minutes in this solution, the embryos were transferred to holding for an average of 5 minutes and then culture “in vitro”. Frozen-thawed morulae, regardless of their quality, were washed in PBS medium and culture in 50µl TCM 199 medium microdroplets at $37^{O}C$, 5%CO_2 and high humidity for 72 hours (fig. 2).

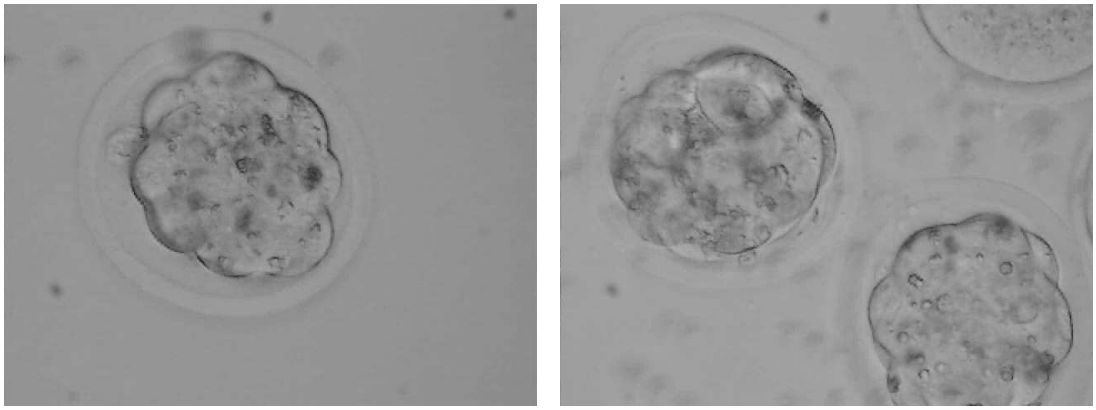


Fig. 2 – Morulae after thawed

Embryos survival was assessed by their ability to develop into expanded, hatching or hatched blastocyst stage (fig. 3).

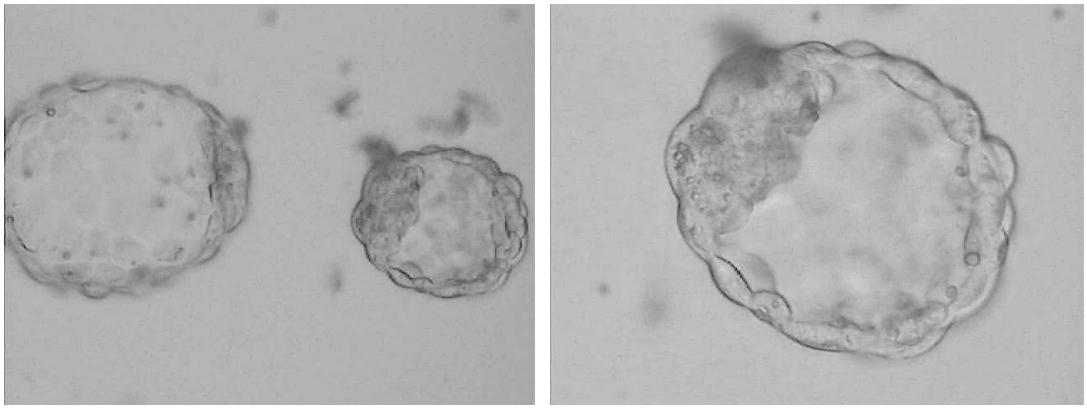


Fig. 3 – Blastocysts obtained after the cultivation of morulae

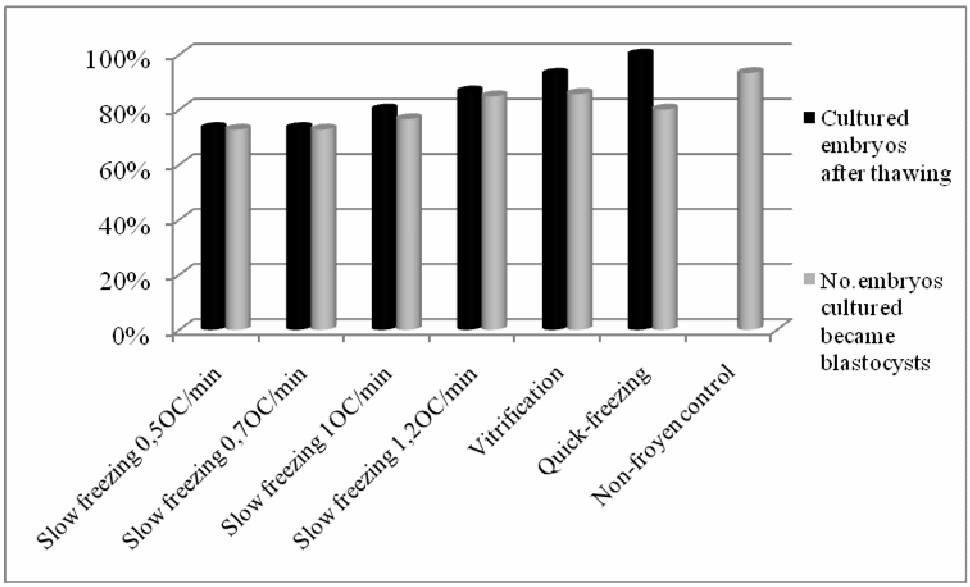
RESULTS AND DISCUSSIONS

From fifteen prepuberal mouse aged 4 – 12 mouths a total of 30 uterine horn were harvested.Total of 145 morulae embryos were collected what it means that we have 9,6 embryos/mouse. After microscopically examination for establish the morphology, only 105 embryos were further used for cryopreservation.

The results obtained in vitro after slow-freezing, vitrification and quick-freezing of mouse compact morulae are presents in Table no. 1.

No.crt.	Treatment	Cryopreserved embryos	Cultured embryos after thawing	Number of embryos cultured that development into blastocyst
1	Slow freezing $0,5^{O}$C/min	15	11 (73,3%)	8 (72,7%)
2	Slow freezing $0,7^{O}$C/min	15	11 (73,3%)	11 (72,7%)
3	Slow freezing 1^{O}C/min.	15	12 (80%)	9 (76,5%)
4	Slow freezing $1,2^{O}$C/min	15	13 (86,6%)	11 (84,8%)
5	Vitrification	15	14 (93%)	12 (85,7%)
6	Quick freezing	15	15 (100%)	12 (80%)
7	Non-frozen control	-	15	13 (93,3%)

Table no. 1 - In vitro survival of mouse embryos after cryopreservation using different methods



Graphic 1 - In vitro survival of mouse embryos after cryopreservation using different methods

Viability of slow-freezing embryos was determined in vitro and is summarize in table no.1. In total 78,3% (47 vs 60) of embryos were recovered after thawing.The percent of embryos that developed in vitro was influenced by the cooling rates.

The viability of embryos cooled at $1,20^{O}$C/minute was significantly higher than $0,50^{O}$C/min. (84,8% vs 72,7%). There was no significant diference in survival rates among 0,5; 0,7 and 10^{O}C/minute (respectively 72,7%, 72,7% and 76,5%). Compared to control, the survival rates of slow-frozen was significantly lower, except for embryos cooled at $1,2^{O}$C/min. (78,7% vs 93,3%).

Total number of 14 vitrified embryos (93,3%) and 15 quick frozen embryos (100%) were recovered after thawing. The survival rates of morulae cryopreserved through vitrification and quick-freezing methods were 85,7% and 80% respectively, and there was no significant difference between them. However, the percent of embryos frozen through quick-freezing methods that developed in culture was significantly lower (82,7%) that control (93,3%).

Numbers of embryos cultured in vitro that develop into blastocyst afther slow-freezing procedures was lower (78,7%) comparative with those who develop after rapid cryoconservation methods (82,7%).

DISCUSSION AND CONCLUSIONS

The study regarding cryopreservation of mouse morulae through different methods shows that:

1. mouse morulae can be slow frozen at rates of 0,5; 0,7; 1 or 1,2^{O}C/min. without considerable reduction of viability. These results are similary to Leibo and Mazur, 1978; Marco Roberto et al., 2001.
2. the results of this study suggest that vitrification or quick freezing can cryopreserve mouse embryos at the morulae stage with more efficiency as a conventional slow freezing procedure. The high in vitro survival rate of morulae after vitrification is comparable to the results of Marco Roberto et al., 2001.
3. slow freezing is an expensive and time-consuming technique for embryos cryopreservation as compared to vitrification and quick freezing. The primary advantages of the vitrification and quick freezing methods for the cryopreservation of mammalian embryos are that the freezing equipment is not necessary and the time required for cooling is reduces.

BIBLIOGRAPHY

1. Marco R., S.Q. Vinicius, 2001, Cryopreservation of mouse morulae through different methods: slow-freezing, vitrification and quick-freezing. Braz. J. Res. Anim. Sci. 38: 160-164.
2. Leibo S.P., P. Mayur, J.C. Daniel, 1978, Methods for the preservation of mammalian embryos by freezing. Academic Press, 179-210.
3. Mukaida T., S. Wada, T. Takahashik, 1998, Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Human Reproduction 13: 2874-2879.
4. Shaw J.M., G.M. Jones, 2003, Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human Reproduction Update, 9: 583-605.
5. Nowshari M.A., G. Brem, 2001, Effect of freezing rate and exposure time to cryoprotectant on the development of mouse pronuclear stage embryos. Human Reprod. 16: 2368-2373.
6. Groza I., T. Trenişan, M. Cenariu, Simona Ciupe, Brîndușa Stegeran, 2004, The cryoconservation of bovine embryos, methods and viability test. Buletin USAMV-CN Cluj- Napoca, 61: 184-194.
7. Ciupe Simona, I. Groza, L. Bogdan, T. Trenişan, M. Cenariu, Brîndușa Stegeran, 2004, Reseach in poliovulation, recovery, assay and medium term in vitro cultivation of mouse embryos. Buletin USAMV-CN Cluj-Napoca, 61: 169-175.