

ECOPHYSIOLOGICAL ANALYSIS SOME ASPECTS FOR THE RESISTING AND VIABILITY OF MOUSE EMBRYOS FREEZING

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ABSTRACT. The aim of this experimental work was to determine if the cryopreservation of mouse blastocysts affect their viability. We used 32 inception, 38 medium and 48 advanced blastocysts. Embryos were obtained by flushing the uterine horns of mouse females in day 4 p.c. (post coitus). "Cryocell-1200" was utilized for cryopreservation of embryos taking into account data from literature. Cryopreservation doesn't affect significantly the viability of preimplantation mouse embryos.

Keywords: Fertilization, embryoculture, cryopreservation, blastocyst

INTRODUCTION

The present work was carried out in order to quantify the extent to which the preservation of the embryos in liquid nitrogen in the mouse blastocyst stage at different degrees of development retain viability after thawing.

After the segmentation of the zygote, is taking place the formation of a blastula consisting of a single type of cells called blastomeres and a cavity filled with liquid.

The blastomeres resulted from the gradual segmentation of the zygote differ in cells with different shapes and functions. Embryonic cells pass mandatory through the development phase: undetermination stage, determination stage and differentiation (GRIGORESCU, 1998).

Undetermination phase is the early phase, when the number of cells is small and blastomeres are identical in terms of having the same capacity for differentiation. Thus, from the stage (2-4 cells) and continue with morula stage (8-16 cells) and with compact morula stage (up to 130-160 cells), the core of the blastomeres are totipotent and equipotent. At this stage, blastomeres regardless of the position, if are detached from each other, evolve until the end of the embryonic development. Each one has the ability to spawn a whole embryo.

Determination phase is characterized by the fact that blastomeres are beginning to occupy certain positions through repeated segmentation, some will expose to the outside, others to the inside. The blastomeres will continue to evolve according to the position occupied in the blastula, according to the relationships between cells, and also due to the ambient factors, gaining a specific functional morphology, some over others. In each cell or group of cells certain protein-enzyme become active, which will initiate the start of specific protein synthesis, which will lead to cell differentiation in reconstruction (phase differentiation).

This is the phase when the embryo arrived at the beginning of the last stage of preimplantational development - blastocyst stage, prepares to leave the pellucid zone by hatching and establishing itself in the lining of uterus.

The time of hatching and implantation of the embryo in the lining of the uterus is, in our view, very important for the smooth development of the organism further on. The blastocyst stage constitute a period of radical changes during the embryo already arrived in the womb and is considered the most suitable for embryotransfer. Because of this, it is important to know the capacity of resistance and embryo viability in cryopreservation after handling them through.

Freezing and thawing of embryos has the advantage that makes it possible to break the flow of embryo-transfer technology in sequences that can operate autonomously. If the freezing and thawing does not significantly affect the viability of embryos, then superovulation and harvesting of the embryos can be made separately from any transfer into their adoptive mothers. This is possible to be performed, because the embryos that are already grown can be untimely preserved in liquid nitrogen until there is a need to transfer them into their own moms.

These are the arguments which led us to estimate such an experiment.

In recent years cryopreservation methods have been improved, becoming more simplified and especially adapted to the conditions of transfer of embryos regarding commercial ways (NIEMANN, 1988).

These were the reasons why in our experiments we have tried to make clear to what extent the freezing and thawing of embryos differently affects embryo viability which have developed in the womb until such a time, were then grown "in vitro", after having been frozen, defrosted and then recultured "in vitro".

MATERIALS AND METHODS

In our experiments we have tried to freeze and defreeze according to the same technology the mouse embryos and to quantify the efficiency of the method. Measure the effectiveness of freezing embryos that we used was the survival rate and reordering of replication of blastomeres and "in vitro" development of embryos after defrosting.

To this end, we've used two variants: in the first variant we've proceeded to freeze embryos immediately after harvesting them from the uterine horns, and in the second variant embryos were cultured in vitro in advance and then frozen.

Embryo freezing was performed using the classical method. For this we've used 32 emerging blastocysts, 38 average blastocysts, and 48 advanced blastocysts (expanded).

The embryos have been collected from the uterine horns of mouse females after 84 hours from the moment of detection of the vaginal stopper.

As a crioprotector for the dehydration of embryos has been used Tampon Phosphate SEF1 freezing medium with 4 g/l D'Ovalbumin + 10% Glycerol.

As a medium of defrosting for the rehydration of embryos has been used Tampon Phosphate SEF 1 medium with 4mg/l D'Ovalbumin + 0,25 M Saccharose.

Freezing was carried out with the help of the freezer "Cryocell-1200" respecting the methodology of the specific literature.

Resistance of preimplantational embryo in the freezing process was estimated using their viability test after thawing.

The percentage of embryos in various degrees of development which have resumed and continued to develop constituted the resistance measure of the embryo towards freezing and thawing.

In vitro cultivation of embryos after defrosting has shown that the process of freezing does not significantly affect the viability of preimplantational mouse embryos. The method used by us proves that preimplantational embryos can be kept for an unlimited period of time.

RESULTS AND DISCUSSIONS

In our experiments we have tried to freeze and thaw mouse embryos by the same technology and to quantify the efficiency of the method. The measurement of the effectiveness of freezing embryos method that we used was the survival rate and replication of the blastomeres and the development of "in vitro" embryos after defrosting.

So in freezing medium, which contains 10% glycerol, as well as in the thaw medium which contains 0,25 M saccharose, the embryos initially contract (Figure 1),

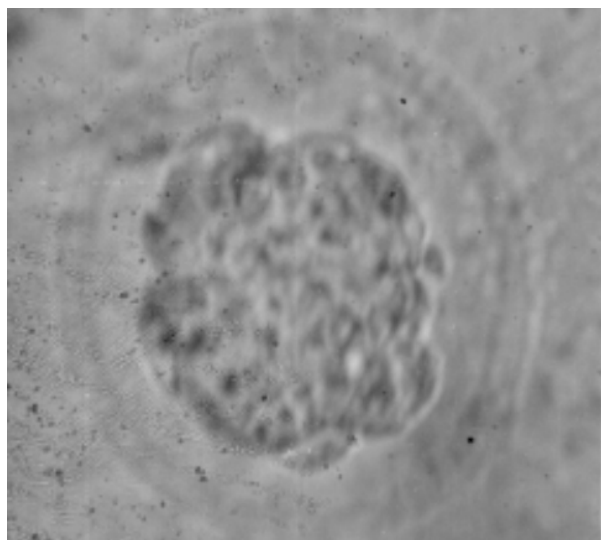


Fig. 1 Contracted blastocyst

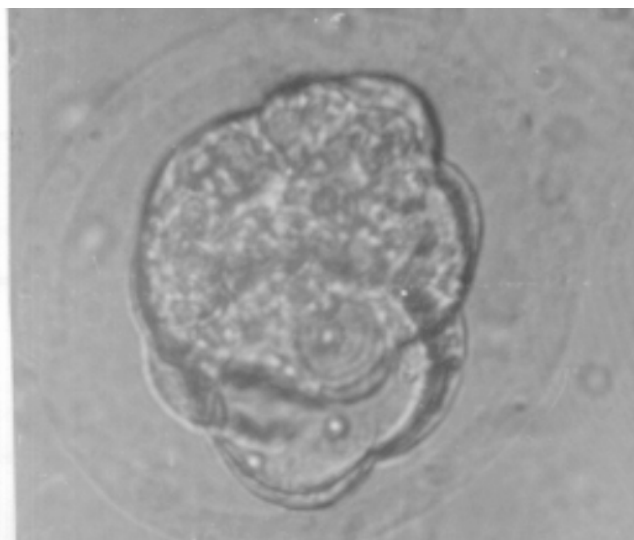


Fig. 2 Blastocyst regaining the initial stage

In our experiments we have used, in the process of freezing and thawing, 118 embryos in various stages of development. We have used therefore 32 emerging blastocysts, 38 average blastocysts and 48 advanced blastocysts. Further on we've frozen them using the technique described in the chapter "material and method of work" immediately after their recollection from the uterus horns of the female mouse. After a week, the embryos were defrosted and deposited in an autoreglable incubator for planting at the temperature of 37°C and an

atmosphere with 5% CO₂ in saturated air and in water vapours.

The culture medium used for this technology has been supplemented with TCM 199 10% calf serum (FCS). At the hatchery of autoreglable incubator in which were lodged for replication Petri plates with grown embryos has been constituted a temperature of 37°C and an atmosphere with 5% CO₂ in saturated air with water vapour.

The results are in table 1 below.

Tabel 1

In vitro development and the hatching of embryo process which have been frozen immediately after their harvesting of the uterine horns

Specification	Nb. of embryos	Nb. of embryos and the ritm of development			% of hatching
		24 ore	48 ore	72 ore	
Early Blastocysts	32	30	26	26	81,3%
Average Blastocysts	38	36	27	27	71,0%
Advanced Blastocysts	48	42	37	37	77,1%
Total	118	108	90	90	76,3%

In the above table it can be seen that all embryos after defrosting resumed it activity of development until they reached the blastocyst stage hatched (Figure 3).

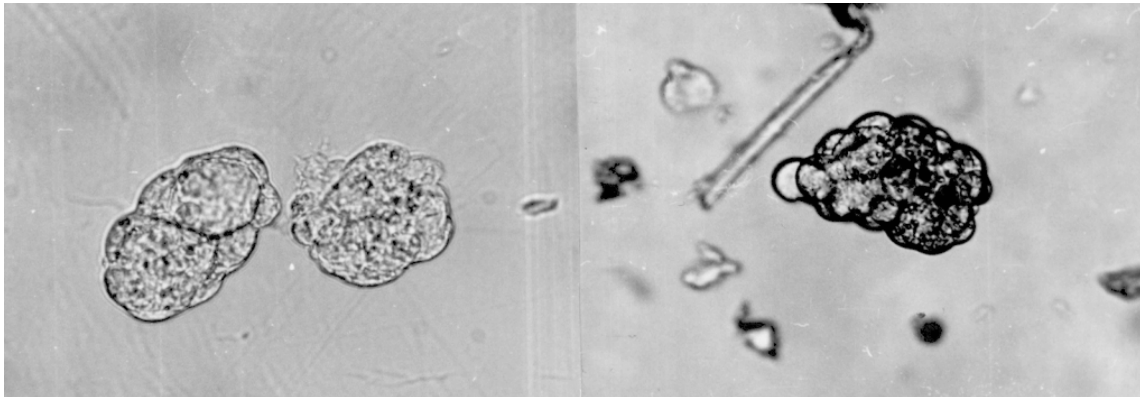


Fig. 3 Hatched Blastocysts (out of zona pellucida) in vitro after having been subjected to the freezing

As it is mentioned above, the second version of the experiment consists in checking the viability of embryos which after harvest have been preliminary cultivated in vitro and then recultivated after cryopreservation.

The embryos of all categories described above have

been frozen and then defrosted using the technology described in the chapter "material and method of work". After defrosting the embryos in all stages of development were cultured in TCM culture medium 199 + 10% FCS to restore viability. The results are in table 2 below.

Tabel 2

In vitro development and the percentage of hatching of frozen and defrosted embryos after previously having been grown in TCM 199 + 10% FCS for 24 hours

Specification	Nb. of embryos	Nb. of embryos and the ritm of development			% of hatching
		24 ore	48 ore	72 ore	
Early Blastocysts	12	12 m.	10 av.	10 ecl.	83,3%
Average Blastocysts	18	18 av.	12 ecl.	12	66,6%
Advanced Blastocysts	30	26 încep.ecl.	24 ecl.	24	80,0%
Hatched Blastocysts	6	4 fix. de f. god	4 alungire	4 mărire vol.	66,6%
total	66	60	50	50	75,8%

The above statements are supported also by comparative results obtained between the two variants of freezing embryos in which I showed the percentage of embryos which after defrosting arrived in "in vitro" culture at the hatched blastocyst stage.

The results above suggests that the freezing and thawing processes, does not impede the further

development of embryos that have been grown after defrosting "in vitro" in TCM 199 + 10% FCS. All frozen embryos that were in the early blastocyst stage and blastocyst stage and resumed their activity in the first 24 hours, and some of them have continued to develop to the expanded blastocyst stage (see Figure 4), after which they walked out of the zona pellucida and hatched.

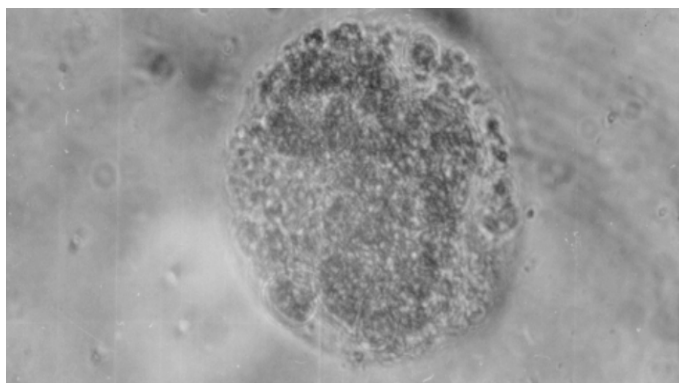


Fig. 4 Embryo in vitro reached the blastocyst stage and exploded after defrosting

83% of emerging blastocysts and 66% of the average blastocysts which have been frozen at this stage have reached the stage of hatched blastocyst after 72 hours of growing.

If we compare these data with those obtained in previous experiments, in which the embryos were frozen immediately after their harvesting in the womb, we note that they resume their activity and develop "in vitro" as if in the process of freezing and storage in deep cold (Cryopreservation), no modification at the level of cells would have occurred.

From the results can be noticed that even the blastocysts at the advanced stage originating from the "in vitro" culture of the average blastocysts, resume their activity reviewing the work of going through further development until the hatched blastocyst stage in a proportion very similar to the development of the young blastocysts, 80-83%. This fact speaks about the

incredible potential of strength and viability of embryos in more advanced stages.

An important aspect which, according to our opinion, deserve to be reported, is the case of embryo cryopreservation in advanced stages, i.e., entrainment of hatched blastocyst in the freezing process. After being harvested from the uterine horns, the embryos in the morula and blastocyst stage have been cultured „in vitro” in optimal conditions. Blastocysts hatched in vitro were sampled from the environment in which they were developed in 24 hours, during which time they managed to get out of the zona pellucida, and to be together with emerging and average blastocysts.

After thawing, the embryos being in hatched blastocyst stage, and regrown resumed their "in vitro" activity and evolved to the stage of elongated embryo and increased its volume (Figure 5).

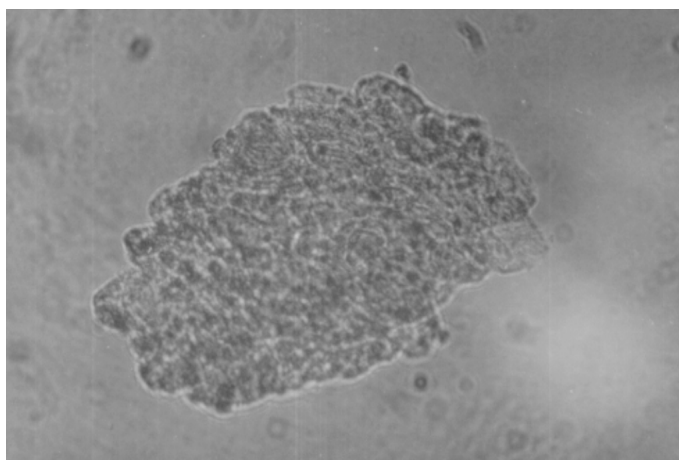


Fig. 5 Hatched Blastocyst, developed in vitro as a result of cryopreservation process

The experiment described in this way gives us the opportunity to believe, that the process of cryopreservation would be an excellent way of keeping indefinitely the embryonic stages of different bodies.

The results causes curiosity, even embryos already out of the zona pellucida and being frozen in this state, survived after defrosting. More so, being regrown, later resumed replication and continued to develop changing their shape and increasing in volume.

Regardless of whether they have hatched "in vitro" or were frozen immediately after their harvesting in the womb, the potential strength and viability of embryos in all stages of the blastocyst has proved to be at a high degree.

The data obtained from the research made us to think that, by cryopreservation, in the future, we could to keep organisms into different embryonic stages, indefinitely even when the embryos are aot of in excess of preimplantational stages.

CONCLUSIONS

The embryos of mouse being in different blastocyst stage with different degrees of development, from early blastocyst and up to blastocyst stage, can be frozen in liquid nitrogen without their viability to suffer too much after defrosting.

The effectiveness of freezing embryos, measured by the development of their subsequent thawing is different, however, is depending on their degree of development.

The process of freezing and thawing of the embryo does not affect negatively their subsequent development, survival rate and hatching for early-stage of blastocyst - 81,3%,, blastocyst - 71% and advanced blastocyst. - 77,1%.

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