Assessment of Survivability of Vitrified Embryos Depending on the Stage of Development

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Abstract

Freezing embryos of excellent and good quality allows you to maintain reproductive potential for patients in the future and achieve pregnancy using one stimulation cycle. Cryopreservation of embryos can be carried out at any stage of development. Vitrification as a method of cryopreservation combines the use of highly concentrated solutions of cryoprotectants and rapid (almost instantaneous) cooling by immersing samples directly in liquid nitrogen. Due to this, the main goal of vitrification is achieved - the absence of the formation of ice crystals, which can damage the cell structure. The advantages of vitrification are as follows: a high concentration of cryoprotectants and a high cooling/thawing rate eliminate the damage caused by the formation of intracellular ice, as well as the undesirable osmotic effects of dehydration and rehydration due to extracellular crystal formation.

Keywords: *Embryo, blastocyst, cell, pregnancy, icm1, te1.*

Introduction

The main criteria for the effectiveness of the vitrification system is the survivability of the embryos. According to the data of the Vienna Consensus (The Viennaconsensus: report fan expert meeting on the development of ART laboratory performance indicators, 2017), the survival rate of blastocysts (embryos of 5-6 days of preimplantation development) is 90% or more[1].

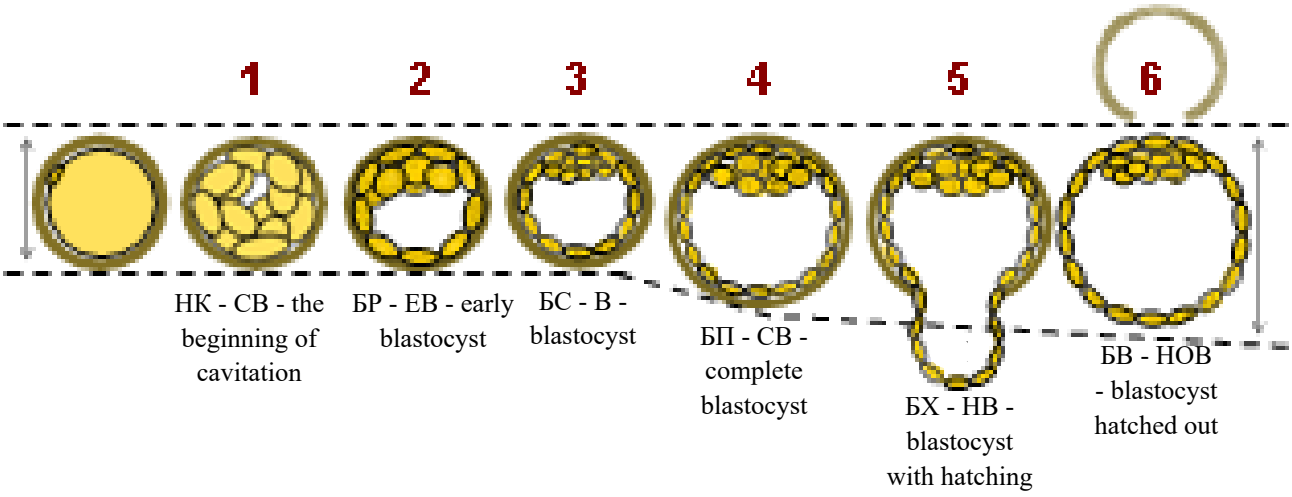
Objective of the Research: Efficiency of various types of carriers for embryo freezing and embryo survivability after thawing under the ART programs.

Materials and Method

The work has used human embryos, the study of which has been carried out in compliance with international ethical and legal standards for the treatment of human embryos [Art. 18 of the Council of Europe Convention on the Protection of Human Rights and the Dignity of the Human Being while Using the Advances in Biology and Medicine, 1997][2]. The study of embryos has been carried out as part of in vitro fertilization cycles at the embryological laboratories of "IDK Medical Company" CJSC. The use of germ cells and human embryos in scientific research has been authorized by the ethics committee of the Samara State Medical University of the Russian Ministry of Health. Embryos have been identified under the control of a stereo microscope (Nikon, Japan). For incubation under conditions of 5% O2, SOOC incubators (Australia) have been used. Vitrolife (Sweden) have been used to cultivate embryos up to 5-6 days of embryonic development. Irvine Scientific environments (USA) and environments of open CryoTop (Japan) and closed CryoTip (USA) types have been used for vitrification of embryos. The fundamental difference between open and closed carriers is the presence/absence of direct contact of the vitrified object with liquid nitrogen. Before freezing, blastocysts of 5-6 days of cultivation have been assessed according to the international classification[6]. Blastocyst assessment takes into account three main parameters: the percentage

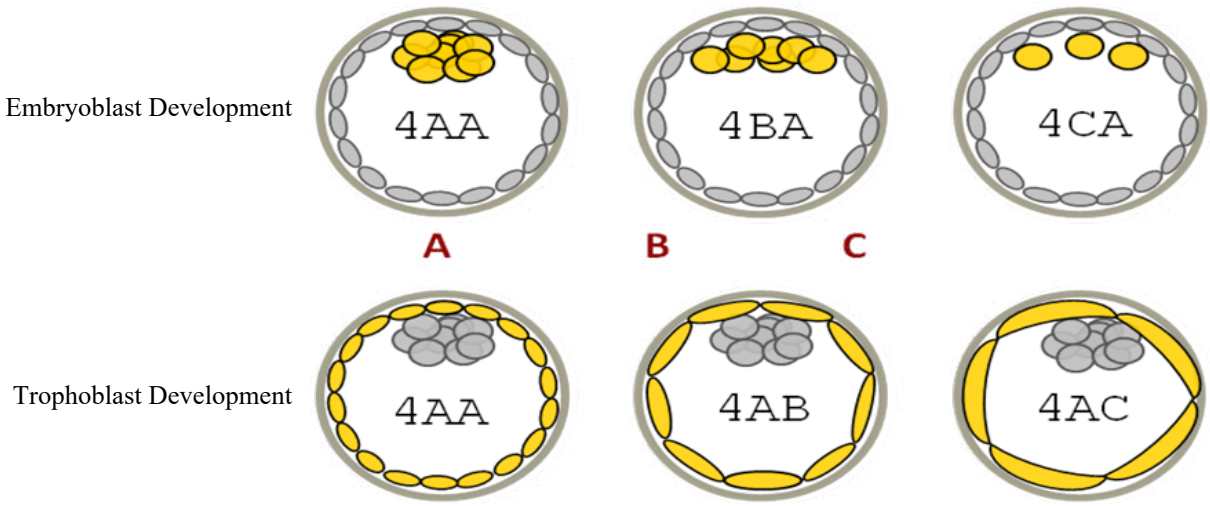
of expansion (increase) of the embryo (blastocyst), the development of internal cell mass and trophectoderm.

Assessment of intracellular mass has been performed under an inverted microscope: (A) densely filled with many cells; (B) weakly grouped with several cells; and (c) very few cells. The following classification has been used for trophectoderm: (A) many cells forming a cohesive epithelium; (B) few cells forming a loose epithelium; and (C) very few large cells. After thawing, the blastocysts have been subjectively assessed according to the degree of repeated expansion of the blastocoel, the degree of cell reorganization, and the degree of cell survivability by two embryologists.



Pic. 1. Blastocyst Assessment by D. Gardner (1999)

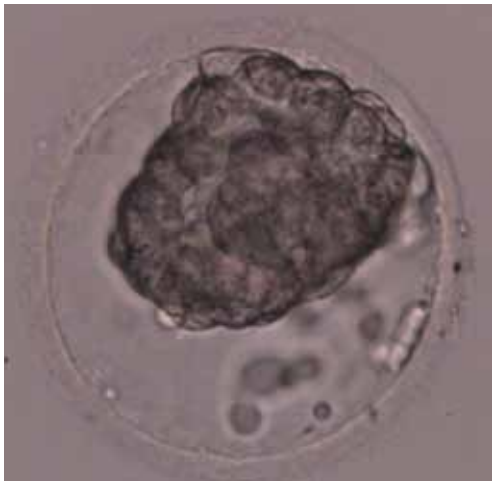
Comment: CB-the beginning of cavitation, EB-early blastocyst, B-blastocyst, CB-complete blastocyst, HB-blastocyst with hatching, HOB-blastocyst hatched out.



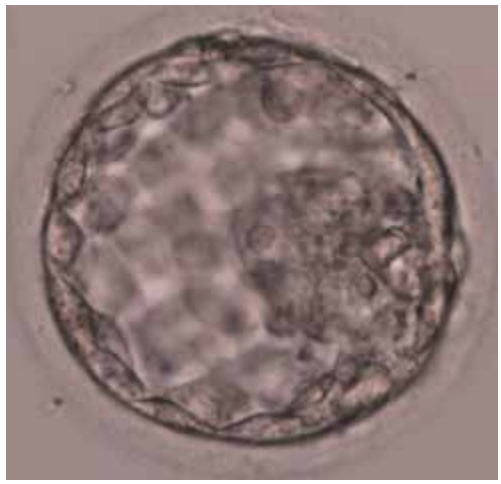
Pic. 1. Blastocyst Assessment by D. Gardner (1999)Illustartions: R.Shafei, 2008

Statistical processing of the results has been performed on a computer in the environment of statistical calculations R (R v.3.5.3, R Studio v.1.1.463), the primary data input has been performed using MS Excel spreadsheets. Descriptive statistics method, tests for comparing proportions, including an exact binomial for small samples and a single-sample proportional test with continuity correction for large samples have been used.

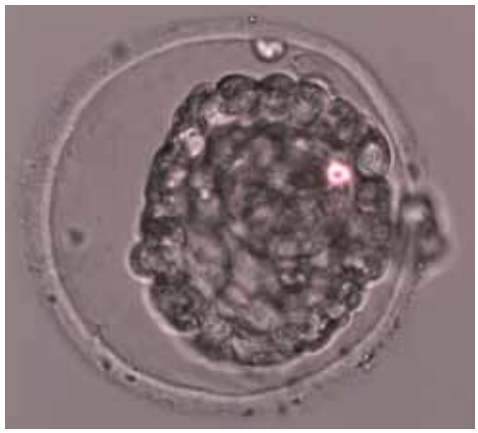
Research Results and Discussion



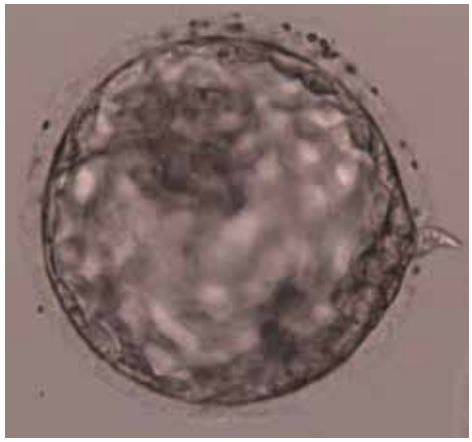
Picture 3. 5-day embryo, stage of collapsed human blastocyst, 10X eyepiece, 20X lens (immediately after thawing)



Picture 4. Blastocyst embryo, 4AA, 10X eyepiece, 20X lens (2 hours after thawing)



Picture 5: 5-day embryo, stage of collapsed blastocyst, 10X eyepiece, 20X lens (immediately after freezing)



Picture 6: Blastocyst embryo, 4AA, 10X eyepiece, 20X lens (2 hours after thawing)

The pictures show embryos immediately after freezing and after 2 hours (Pic. 3-6). 1-2 hours are needed to restore the blastocyst and to morphologically assess its viability. If the embryo has more than 50% intact cells, then it is considered viable.

Table №1. Embryo survivability depending on embryo developmental stage

No.	Embryo development stage	ICMdevelopment stage	Trophectoderma development stage	Number of embryos	Survivability, %
1	0.morula	<NA>	<NA>	5	100.00
2	1.beg.of cav.	<NA>	<NA>	156	90.33
3	2.earl.blast	A.icm1	A.te1	1	100.00
4	2. earl.blast	<NA>	<NA>	187	90.00
5	3. blast	A.icm1	A.te1	41	100.00
6	3. blast	A.icm1	B.te1	109	95.51

No.	Embryo development stage	ICMdevelopment stage	Trophectoderma development stage	Number of embryos	Survivability, %
7	3. blast	A.icm1	C.te1	3	100.00
8	3. blast	B.icm1	A.te1	8	100.00
9	3. blast	B.icm1	B.te1	141	89.33
10	3. blast	B.icm1	C.te1	27	92.16
11	3. blast	C.icm1	C.te1	2	100.00
12	3. blast	<NA>	<NA>	1	NA
13	4. blast	A.icm1	A.te1	193	96.96
14	4. blast	A.icm1	B.te1	168	97.90
15	4. blast	A.icm1	C.te1	1	100.00
16	4. blast	A.icm1	<NA>	1	100.00
17	4. blast	B.icm1	A.te1	21	100.00
18	4. blast	B.icm1	B.te1	107	97.21
19	4. blast	B.icm1	C.te1	13	100.00
20	4. blast	C.icm1	B.te1	1	100.00
21	4. blast	C.icm1	C.te1	1	100.00
22	4. blast	<NA>	<NA>	1	100.00
23	5. blast	A.icm1	A.te1	43	96.43
24	5. blast	A.icm1	B.te1	15	100.00
25	5. blast	B.icm1	A.te1	7	100.00
26	5. blast	B.icm1	B.te1	8	81.82
27	6. blast	A.icm1	A.te1	5	100.00
28	6. blast	A.icm1	B.te1	4	75.00
29	6. blast	B.icm1	B.te1	3	100.00
30	<NA>	<NA>	<NA>	133	37.68
	Total			**1406**	**94.49**

From table 1 it follows that a total number of 1406 embryos have been analyzed at different stages of development. The embryo survival range is quite wide and ranges from 75 to 100%. It should be noted that embryos of the 6th degree of expansion, namely, hatched blastocysts, demonstrate the lowest survival rate. Probably this is due to the lack of a fertilization membrane and the cytotoxic effect of cryoprotectors on trophectoderm cells. The number of embryos in this group is extremely small to draw any unambiguous conclusions on this subject.

In more numerous groups (more than 100 cases), the survival rate has been more than 95%, which indicates a high degree of cryotolerance of excellent and good quality embryos. Nevertheless, we considered it necessary to conduct a further, more detailed analysis of the dependence of the frequency of pregnancy on the stage of the frozen blastocyst (degree of expansion, the severity of ICM and TE). On the basis of the degree of expansion, the indicators of the pregnancy rate (PR, positive ultrasound) increase with an increase in the degree of expansion from the stage of onset of cavitation of the blastocyst to increased blastocyst (degree of expansion 4), reaching a maximum in hatched blastocysts lacking a fertilization membrane. This trend is quite natural: an embryo with a higher level of organization and cell differentiation gives higher PR indices. However, the decline in the blastocyst group with the 5^{th} expansion degreeis noteworthy; these are embryos that have been frozen during hatching. Apparently, embryos of this developmental stage are the

most vulnerable to the effects of the freezing procedure. The difference in the proportion of positive ultrasound has significantly differed between the stages ($p < 0.01$). That is, the percentage of PR-positive ultrasound scan has been growing from morula (20.0%) to 6-blast (66.7%) with a slight "failure" at the stage of expanded [5] blastocysts (37.5%). Carrying out a comparative analysis according to the following criterion — the degree of development of intracellular mass (ICM), we have obtained the expected results: the higher the degree of compaction of cells in the ICM, the higher the PR indices. In blastocysts with ICM A (581 embryos), the PR rate has been 51.3%, in blastocysts with ICM B (334 embryos) - 39.8%. The differences are significant at $p = 0.004$. It should be noted that in the group of blastocysts with intracellular mass C (there are extremely few or no cells), we have received 50% of PR. This indicator can hardly be considered objective due to the extremely small group (4 embryos), since embryos with a similar morphology, as a rule, are not supposed to be frozen. The pronouncement of trophectoderm, the degree of development of which is most significant in the process of implantation, directly correlates with the PR data. When analyzing 918 embryos, we have found that blastocysts with class A trophoctoderm (318) give the most indicators - 57.5% of PR, blastocysts with class B trophoctoderm (553) - 42.9% of PR, with class C trophoctoderm - 27.7% of PR. These differences are significant at a level of $p < 0.01$.

To assess the effectiveness of different carriers, the following criteria have been taken into account: survival rate, average transfer embryo/s score, average number of transfer embryos, pregnancy incidence rate, birth rate and loss (table 1).

Table 1. The main criteria for the effectiveness of the cryoprogram of embryos between open and closed types of carriers

	Closed carrier (CryoTip)	Open carrier (CryoTop)	p
Average age of the patient, years	34,1	33,5	
Number of thawed embryos	611	678	
Survivability, %	84,8%	95,1%	<0,0001*
Average score of embryos/transfer	3,9	4,1	
Average number of embryos/transfer	1,2	1,3	
PR, % (per transfer)	39,5	44,2	0,001*
Birth rate, % (per clinical pregnancy)	72,7	67,3	0,003*
Loss, % (per clinical pregnancy)	27,3%	24,3%	0,044*

In 2015-2016, 1289 embryos were thawed on the types of carriers under consideration. Embryo survivability on closed carriers has been 84.8%, on open - 95.1%, and this difference is statistically significant ($p < 0.0001$). The average transfer embryo score in group I has been 3.9, in group II - 4.1. The average number of embryos per transfer is 1.2 and 1.3, respectively. The pregnancy rate has been statistically significantly different ($p < 0.0001$) and has amounted to 39.5% (group I) and 44.2% (group II). The birth rate for clinical pregnancy, taking into account the known outcomes, has been 72.7% in group I, 67.3% in group II, and losses have been 27.3% and 24.3%, respectively. Birth rates and losses have a statistically significant difference.

Conclusion

Thus, conducting a comparative statistical analysis, the influence of all components of the blastocyst assessment—the stage, the degree of ICM development, and the pronouncement of trophectoderm—on the degree of survival and the level of pregnancy rate, has been disclosed. Embryos with the highest level of development of intracellular mass A and trophectoderm A give the highest results[3]. Unexpected results have been obtained on the PR in the group of embryos with intracellular mass C (50%), they can hardly be considered natural given the small number of cases (4).[4] The degree of expansion of blastocysts also shows a

direct relationship with PR, with the exception of stage 5 preimplantation development blastocysts (blastocysts with hatching). Apparently, this energetically active process for the embryo (when the embryo expands at regular intervals) is extremely important. The embryo is trying to find the most favorable place for rupture of the membrane of fertilization and exit from it. In fact, this stage of development is a kind of critical period of development for the embryo[5]. Accordingly, at this moment, it seems to be the most vulnerable to the impact of internal and external factors. Vitrification in a certain way at this moment is the very external factor that determines lower PR indices in embryos of this developmental stage[6].

According to the results of the analysis, the use of open carriers in cryopreservation allows us to conclude about a higher level of embryo survivability. Most likely this is due to direct contact of the object with liquid nitrogen and a high freezing rate, which can be a decisive factor in determining the success of vitrification on an open carrier. Clinical indicators (incidence of clinical pregnancy, childbirth and pregnancy loss), which directly depend on the level of survivability, also demonstrate a statistically significant difference in favor of using open carriers in embryological practice[7].

Ethical Clearance: No ethical approval is needed.

Source of Funding: Self

Conflict of Interest: Nil

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