

INVESTIGATING THE RELATIONSHIP BETWEEN
THE DAY OF EMBRYO FREEZING
AND PREGNANCY RATES

Nadya Petrova^{*,**}, Elena Hristova^{*}, Marina Hristova^{**},
Plamen Todorov^{*,**}

Received on July 25, 2022

Presented by R. Pankov, Member of BAS, on September 27, 2022

Abstract

In the last decades, infertility incidence is continuously increasing, reaching to about 10–15% of the couples of reproductive age. As more patients turn to assisted reproductive technologies, there is an ongoing search for the development of new techniques and improvement of the existing ones in the field. Frozen embryo transfer is one of the approaches that are widely used in recent years, because of the advance in the cryopreservation procedures. Still, there is an ongoing debate whether the embryos should be frozen on day 3, 4 or 5, before culture and transfer. The present study is a retrospective analysis of the clinical pregnancy rates in the period of 2018–2021, in “In vitro OB Medical Centre Dimitrov”, depending on which stage the embryo is cryopreserved. Our results point out that there are significantly higher pregnancy rates of day 3 and day 4 frozen embryos, compared to day 5.

Key words: embryo cryopreservation, vitrification, blastocyst, Frozen Embryo Transfer (FET)

Introduction. Infertility is a socially significant disease that affects at least one in ten couples of reproductive age [1]. The procedures performed in the fertility clinics, like in vitro fertilisation (IVF) and intracytoplasmic sperm injection

The present research has been performed under grant KP-06-N51/11.
DOI:10.7546/CRABS.2022.12.06

(ICSI) often require the cryopreservation and subsequent transfer of thawed embryos. The first pregnancy after frozen embryo transfer (FET) was reported in 1983 [2]. Nowadays, after the improvements in laboratory techniques, the number of FET treatments has exceeded that of conventional IVF and furthermore in some countries more children are born after FET than after fresh embryo transfer [3].

The reasons justifying the decision to cryopreserve embryos are various, but among the most important are the inability to perform transfer on the days after fertilization due to medical implications (risk of the development of ovarian hyperstimulation syndrome, endometrial thickness of less than 7 mm, appearance of fluid in the uterine cavity, different pathologies of the endometrium) and the fact that more good quality embryos have been obtained, than can be transferred. During assisted reproductive technologies (ART) no more than 1–3 embryos are transferred per cycle, in order to avoid multiple pregnancies, which are linked to a higher risk of medical complications, both for the mother and the child. With the aim to minimize that potential hazard, in many countries there are regulations imposed for the number of embryos that can be transferred per cycle, as for example, in France and Sweden they are maximum two. In the Bulgarian legislation there are specific criteria to be considered in every individual case, like the age of the female patient, number of failed IVF/ICSI procedures, embryo quality. Some ART centers have chosen the policy of elective single embryo transfer [4].

Among other reasons for the cryopreservation of embryos during the procedures of assisted reproduction are the need of the couples to undergo additional time-consuming tests, as preimplantation genetic diagnosis, or to turn to fertility preservation strategies for patients with upcoming radio- or chemotherapy, etc.

In recent years, with the improvement of the cryotechnologies and the introduction of vitrification as a method there is an increase in the viability of thawed embryos. Moreover, some studies show that the success rate of the frozen embryo transfer (FET) is following that of non-cryopreserved ET [5,6].

Although the cryopreservation procedures are already well established in ART clinical practice, there is still an ongoing debate on the optimal day of development the embryos should be frozen. Considering this, the aim of the present study is to assess the obtained results from FET (percentage clinical pregnancies) in our ART clinic in relation to the day of cryopreservation of the embryos (on the third, fourth or fifth day). It is expected that the data will be useful in the optimization and the standardizing of the FET and cryopreservation protocols.

Materials and methods. Study design. This is a retrospective study of the results obtained in “In vitro OB Medical Centre Dimitrov” during 2018–2021. A total of 173 FET on day 5, 165 patients (in some cases one woman have had two FET cycles) have been analyzed. The FET were separated into three groups, depending on the day of freezing: group 1 ($n = 43$) – frozen on day 3, thawed and cultured until the blastocyst stage before transfer; group 2 ($n = 89$) – cryopreserved on day 4 and cultured to the blastocyst stage after warming and

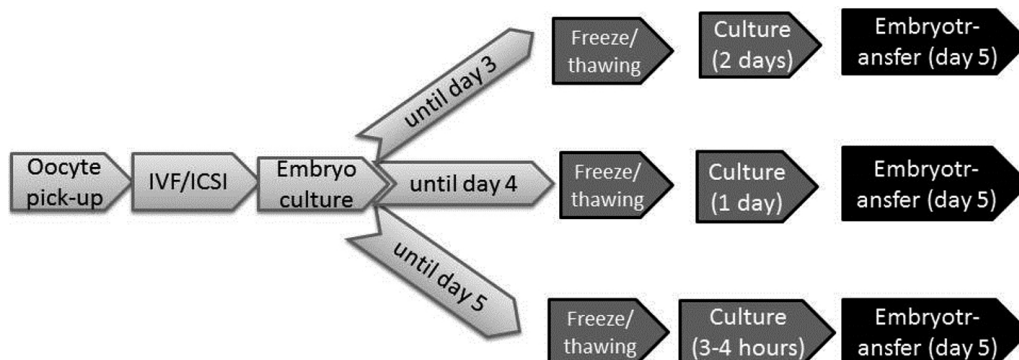


Fig. 1. A schematic representation of the experimental design

group 3 ($n = 41$) – frozen on day 5 and cultured for 3 to 4 h before transfer. The study design is shown in Fig. 1.

Patients over 37 years of age and those with repeated implantation failures have been excluded from the evaluation. Donor oocytes were not included in the investigation, as well.

Obtaining of the gametes, fertilization and embryo culture. Standard protocols for ovarian stimulation have been used. The oocytes have been pick-up by transvaginal puncture of ovarian follicles under ultrasound control. Fertilization was with either IVF or ICSI and the obtained embryos were cultured in sequential media (Vitrolife or Origio).

Assessment of the embryos. The embryos were evaluated on a daily basis for their quality and only good quality ones have been cryopreserved [7].

Freezing procedure. The embryos were frozen on day 3 (D3), 4 (D4) and 5 (D5) post-insemination with vitrification in open-system. Not more than three embryos were placed onto the device. Ready-to-use commercially available media were used (Vitrification solution, Kitazato) under the manufacturer's guidelines [8]. Collapse of the expanded blastocysts was performed prior to cryopreservation.

Thawing and culture. The embryos were thawed in ready-to-use medium (Thawing solution, Kitazato), according to the manufacturer's instructions. After warming, they were cultured to the blastocysts stage. Only good quality embryos were chosen for transfer.

Embryotransfer. The catheter for embryo-transfer was introduced into the uterus under ultrasound control. After the procedure, it was inspected under a microscope to make sure that all the embryos have been placed.

Statistical analysis. Pregnancy was confirmed by detection of cardiac activity of the embryos under ultrasound inspection.

The statistical significance was assessed using Chi-squared test. A probability of $p < 0.05$ was considered significant.

Results. In the present investigation, 469 embryos obtained from 165 women aged 22 to 37 years were included. In some of the patients, two FET have been performed.

A total number of 469 thawed embryos were included in the investigation, 116 of them frozen on D3, 258 – on D4 and 95 on D5. Overall there was no difference in their quality, depending on the developmental stage at the time of cryopreservation. Morphologically, the embryos have retained their integrity on (D3) 92.5%, (D4) 93%, and (D5) 91.5% (Table 1).

T a b l e 1

Data from the analysis (There is no difference between D3 and D4.

*It is significant between D3 and D5, as well as between D4 and D5)

	D3	D4	D5	Significance
Avarage age of women	33.2	32.8	34.2	$p > 0.05$
Thawed embryos	116	258	95	
Percentage of embryos surviving thaw	92.50%	93%	91.50%	$p > 0.05$
Number of FET	43	89	41	
Rate of clinical pregnancies	48.8%	49.4%	26.80%*	$*p < 0.05$ $p > 0.05$

In the course of the investigation, on day 5 173 FET have been performed. Of them, 43 of the embryos were thawed on D3, 89 on D4 and 41 on D5 (Table 1). Not more than two embryos have been returned in each transfer procedure. In seven of the patients, the FET was not performed, because the embryos have stopped their development or lost viability after thawing. Representative images of embryos cultured to the blastocyst stage after cryopreservation are shown in Fig. 2.

In the current investigation, we have analyzed the success rate of the FET, depending on the stage at which the embryos have been vitrified: D3, D4, or D5. The observed average pregnancy rate was 43.9%, for D3 frozen embryos – 48.8%, D4 – 49.4% and D5 – 26.8%. Statistically significant difference in the clinical pregnancies ($p = 0.038$) as shown by the Chi-squared test was detected between the embryos cryopreserved on D3 and D5 and between D4 and D5 ($p = 0.015$). No difference was observed between D3 and D4 frozen embryos (Table 1 and Fig. 3).

Discussion. In the last decade, the significant improvement of the ready-to-use cryopreservation media and the techniques allowed for the increase in the success rate of freezing embryos at all developmental stages – from pro-nuclear to blastocyst. In clinical practice, vitrification on D3 or D5 is most widely used. Vitrification (from Latin vitreus – glass) is a method of superfast cooling, in which the formation of intracellular crystals is avoided, because the water molecules freeze in a glass-like structure. It is routinely used in the preservation of oocytes and embryos in human ART. A typical vitrification protocol includes the placement of the cells or embryos in equilibration solution containing agents in lower concentration

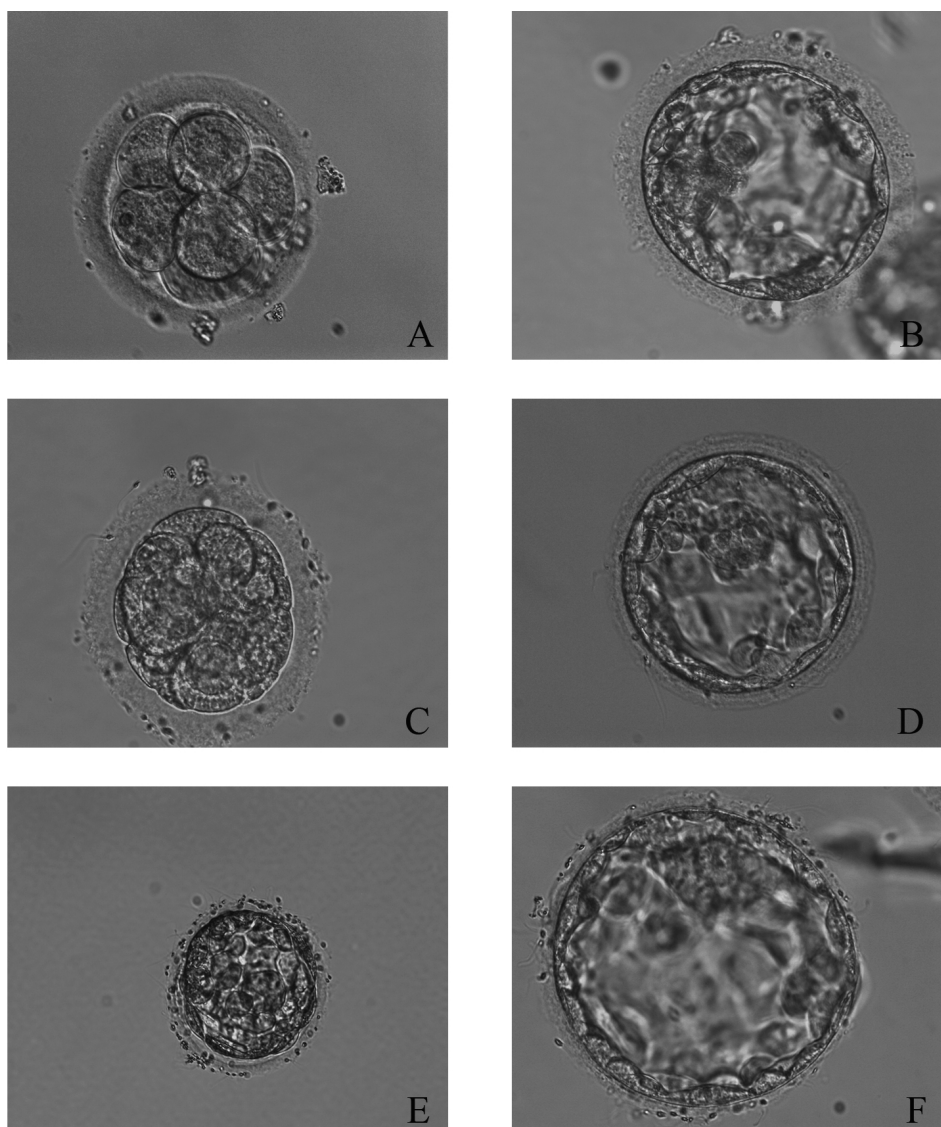


Fig. 2. Thawed embryos: A) vitrified on day 3; B) vitrified on day 3 and cultured for two days to blastocyst; C) cryopreserved on day 4; D) cryopreserved on day 4 and cultured for two days to blastocyst; E) vitrified on day 5; F) thawed embryo vitrified on day 5 and cultured for 4 h

for 10 to 15 min (7.5% DMSO and 7.5% ethylene glycol) and subsequent transfer into vitrification solution (15% DMSO, 15% ethylene glycol, 0.5 M sucrose) for 60 to 90 s [9]. After the saturation of the bio-objects with cryoprotectants they are placed on a carrier device and plunged directly into liquid nitrogen.

As mentioned above, vitrification can be used to preserve embryos at different developmental stages, as all these approaches have their advantages and

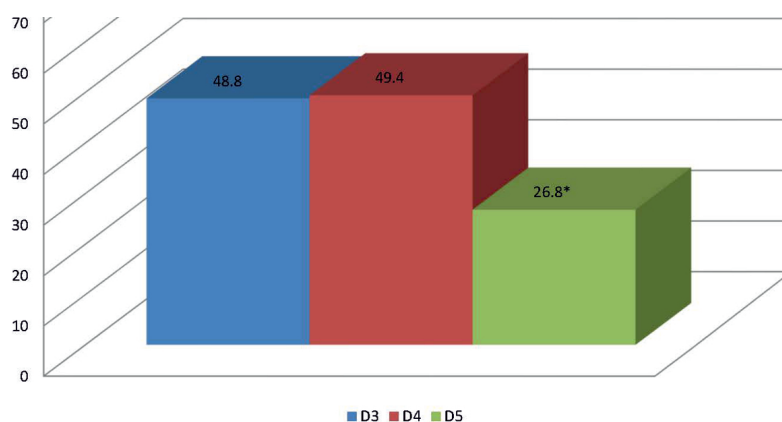


Fig. 3. Pregnancy rate according to the day of cryopreservation * $p < 0.05$

drawbacks, not only from biological, but also from organizational and financial point of view.

Freezing embryos at earlier stages provides more time for their culture after thawing and to assess their quality before transfer, which is the strategy preferred in some ART clinics [10]. In this way, the implantation rates may be improved and moreover, data exist that it also decreases the risk of miscarriage, ectopic and multiple pregnancies [11,12]. In some cases, however, due to organizational reasons freezing of D3 or D4 embryos cannot be carried out. For example, when preimplantation genetic diagnosis is needed, in order to avoid the risk of mosaicism, trophectoderm biopsy is preferred and the embryos are kept up to D5 [13].

Cryopreservation at the blastocyst stage is challenging, because of the presence of the blastocoel and their multicellular structure. These characteristics expand the technological time that the embryos need to spend into the vitrification media to become saturated and, respectively, they are exposed for longer to their toxic activity. The larger amount of fluid in the expanded blastocysts increases the risk of ice-crystal formation, which can impair the integrity of the cells. To avoid damage, the so-called “collapse” of the embryos has to be performed. It is an artificial constriction of the blastocoel with the use of a microneedle or laser impulse before the cooling procedures [14]. An advantage of vitrification at the blastocyst stage is that the embryos could be cultured for a longer period, which allows for better selection of the ones with superior quality before the freezing process. Financially, that reduces the costs for cryopreservation media, storage, thawing media and the culture afterwards of the “not promising” embryos. In addition, data exist that the survival rate of blastocysts after thawing is higher than for cleavage stage embryos [15]. In our study, we did not observe statistical difference with respect to recovery after thawing of D3, D4 and D5 embryos.

Regarding the implantation and clinical pregnancy rates with the day of cryopreservation, the published reports are controversial. According to some authors,

there is no statistically significant difference between the stage of development at which the embryos are frozen and the success rate [16,17]. A possible reason for this data is that some of the transfers of thawed embryos has been done at an earlier stage rather than culture them to blastocysts after warming. One of the above groups, observed the highest clinical and ongoing pregnancy rate with embryos frozen on D4 and transferred on D5, which corresponds with our results [16]. Other papers also show that the cryopreservation on D3 and the subsequent culture to blastocyst stage before transfer increases the pregnancy rate [18]. Significantly higher clinical pregnancy rates for D4 compared to D3 vitrified embryos have been reported by KAARTINEN et al. [19], which is not consistent with our findings. Furthermore, the authors show similar trend in the results for embryos cryopreserved on D4 and D5, and our investigation points out that there is a statistical difference between those experimental groups.

Conclusion. The obtained results in the present investigation indicate that the optimal period for cryopreservation of embryos in ART procedures is on day 3 or 4, which gives enough technological time to select the best quality embryos both before and after cryopreservation.

REFERENCES

- [1] LAWRENZ B., C. COUGHLAN, L. MELADO, H. M. FATEMI (2020) The ART of frozen embryo transfer: back to nature!, *Gynecol. Endocrinol.*, **36**(6), 479–483.
- [2] TROUNSON A., L. MOHR (1983) Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo, *Nature*, **305**, 707–709.
- [3] TERHO A. M., S. PELKONEN, R. TOIKKANEN, S. KOIVUROVA, J. SALO et al. (2021) Childhood growth of term singletons born after frozen compared with fresh embryo transfer, *Reprod. Biomed. Online*, **43**(4), 719–726.
- [4] REIMUNDO P., J. M. ROMERO, T. PÉREZ, E. VEIGA (2021) Single-embryo transfer: a key strategy to reduce the risk for multiple pregnancy in assisted human reproduction, *Advances in Laboratory Medicine / Avances en Medicina de Laboratorio*, **2**(2), 179–188.
- [5] WONG K. M., S. MASTENBROEK, S. REPPING (2014) Cryopreservation of human embryos and its contribution to in vitro fertilization success rates, *Fertil. Steril.*, **102**(1), 19–26.
- [6] ROQUE M., T. HAAHR, S. GEBER, S. C. ESTEVES, P. HUMAIDAN (2019) Fresh versus elective frozen embryo transfer in IVF/ICSI cycles: a systematic review and meta-analysis of reproductive outcomes, *Hum. Reprod. Update*, **25**(1), 2–14.
- [7] Alpha Scientists in Reproductive Medicine (2011) *Reprod. Biomed. Online*, **22**(6), 632–646.
- [8] https://www.kitazato-ivf.com/wp-content/uploads/2021/05/Brochure_Vitrification_Protocol_v_2019-1.pdf.
- [9] MORI C., A. YABUCHI, K. EZOE, N. MURATA, Y. TAKAYAMA et al. (2015) Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solu-

- tions for embryo vitrification in human assisted reproductive technologies, *Reprod. Biomed. Online*, **30**(6), 613–621.
- [10] ALBAHLOL I., M. GHANEM, L. ELBOGHDADY, E. SEDEEK, Y. MESBAH et al. (2022) Extended culture of cleavage embryo to blastocyst embryo is among the good predictors of successful outcome in vitrified-thawed ICSI cycles, *Middle East Fertil. Soc. J.*, **27**, 13.
 - [11] HOLDEN E. C., B. N. KASHANI, S. S. MORELLI, D. ALDERSON, S. K. JINDAL et al. (2018) Improved outcomes after blastocyst-stage frozen-thawed embryo transfers compared with cleavage stage: A Society for Assisted Reproductive Technologies Clinical Outcomes Reporting System study, *Fertil. Steril.*, **110**(1), 89-94.e2.
 - [12] KRISHNAMOORTHY K., P. GREENBERG, B. PERLMAN, S. MORELLI, S. JINDAL et al. (2021) The incidence of ectopic/heterotopic pregnancies after blastocyst-stage frozen-thawed embryo transfers compared with cleavage-stage: a SART CORS study, *Fertil. Steril. Rep.*, **2**(4), 421–427.
 - [13] AOYAMA N., K. KATO (2020) Trophoctoderm biopsy for preimplantation genetic test and technical tips: A review, *Reprod. Med. Biol.*, **19**(3), 222–231.
 - [14] MUKAIDA T., C. OKA, T. GOTO, K. TAKAHASHI (2006) Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts, *Human Rep.*, **21**(12), 3246–3252.
 - [15] GRYNBERG M., P. PATRIZIO (2022) *Female and Male Fertility Preservation*, Springer Nature Switzerland, ISBN 978-3-030-47766-0.
 - [16] KORKMAZ C., Ü. GÜL YILDIZ, U. FIDAN, B. BAYKAL, S. CEYHAN et al. (2020) Investigation of transfer results of human embryos that were vitrified and thawed at the cleavage, morula and blastocyst stages, *Zygote*, **28**(3), 191–195.
 - [17] RAO J., F. QIU, S. TIAN, Y. YU, Y. ZHANG et al. (2021) Clinical outcomes for Day 3 double cleavage-stage embryo transfers versus Day 5 or 6 single blastocyst transfer in frozen–thawed cycles: a retrospective comparative analysis, *J. Int. Med. Res.*, **49**, doi:10.1177/03000605211062461.
 - [18] RAHAV-KOREN R., S. INBAR, N. MILLER et al. (2021) Thawing day 3 embryos and culturing to day 5 may be a better method for frozen embryo transfer, *J. Assist. Reprod. Genet.*, **38**(11), 2941–2946.
 - [19] KAARTINEN N., K. KANANEN, C. TOMÁS, H. TINKANEN (2017) Day 4 embryos should not be underestimated in IVF, *Gynecol. Reproduct. Endocrinol.*, **1**(1), 35–41.

**Institute of Biology and Immunology
of Reproduction
Bulgarian Academy of Sciences
73 Tsarigradsko Shosse Blvd
1113, Sofia, Bulgaria
e-mails: hristova.elena@gmail.com
plamen.ivf@gmail.com
crustacea@abv.bg*

***“In vitro OB Medical Centre Dimitrov”
2 Dr. Stoyan Chomakov St
Mladost 1 district,
1750 Sofia, Bulgaria
e-mail: mhristova_abv.bg*