

A comparative study of post-warming survival rates and clinical outcomes of human blastocysts vitrified/warmed by CryoTouch and Cryotop methods

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ABSTRACT

Objective: Advances in embryo culture conditions and the development of vitrification as a revolutionary cryopreservation method have allowed for routine use of blastocyst transfer in assisted reproduction technology (ART) cycles. Several vitrification/warming media and devices have been introduced for commercial use so far. The aim of this retrospective study was to compare post-warming survival rates and clinical outcomes of human blastocysts vitrified/warmed by two different commercial methods (CryoTouch and Cryotop) during ART cycles.

Methods: This retrospective study assessed a total of 50 frozen embryo transfer (FET) cycles conducted on 56 warmed blastocysts between January 2018 and December 2020. Post-warming blastocyst survival rates and clinical outcomes including clinical pregnancy and live birth rates were calculated after single blastocyst transfer cycles.

Results: The results revealed no significant differences between two groups in post-warming survival rate (p -value=0.8381), clinical pregnancy rate (p -value=0.8157) and live birth rate (p -value=0.7041).

Conclusions: Post-warming survival rates and clinical outcomes were comparable with no significant difference in blastocysts vitrified/warmed by CryoTouch and Cryotop commercial methods.

Keywords: cryopreservation, embryo vitrification, survival rate, clinical outcome

INTRODUCTION

Successful clinical pregnancies with cryopreserved embryos were first reported in early 1980s (Al-Azawi *et al.*, 2013). Ever since, cryopreservation has become a routine method in infertility clinics as it gives the opportunity to transfer the frozen/warmed embryos to uterus whenever the endometrium is optimally prepared. Over the past decades, the techniques of embryo cryopreservation have evolved to heighten the post-warming survival rate and clinical outcomes (Al-Azawi *et al.*, 2013). Currently, vitrification and slow freezing are the available methods for embryo cryopreservation (Serdarogullari *et al.*, 2019). During vitrification, ice crystal formation and growth are avoided by ultrafast freezing of the embryonic cells using a simple procedure without needing any costly device (Serdarogullari *et al.*, 2019), while slow freezing requires expensive equipment and more time. Due to the ease of use, rapid procedure and low cost of vitrification, it has become the method of choice worldwide and nearly all of the clinics utilize this method for cryopreservation of

embryos in any developmental stage (Fasano *et al.*, 2014; Wirleitner *et al.*, 2016), and there are multiple reports of improved post-warming embryo survival rate and clinical outcomes using vitrification for human cleavage and blastocyst stage embryos in comparison to slow freezing (Cobo *et al.*, 2012).

As superior clinical results of blastocyst transfer over cleavage-stage embryo transfer have been revealed by a number of investigators (Wang *et al.*, 2021), there is an increasing trend towards extended culture of embryos until blastocyst stage. Nowadays, the single blastocyst transfer strategy has become popular based on improved clinical results (Wang *et al.*, 2021). After transferring the selected blastocyst to the uterus, surplus blastocysts must be vitrified for later use. The blastocyst is sensitive to ice crystal formation during vitrification as the blastocoele cavity contains a large amount of water. Therefore, blastocyst vitrification must be performed carefully to reduce the risk of ice crystal formation.

The quality of blastocysts, duration of exposure to cryoprotectants and volume of the solution used for loading the embryos on the vitrification device largely affect the survival rate after warming and the subsequent clinical results (Zeng *et al.*, 2018). In addition, the composition of vitrification/warming media (solutions) and vitrification device have massive effects on the final results. Today, the use of vitrification/warming media and device from the same manufacturer is more common and investigators tend to use the term "vitrification method" when performing vitrification/warming procedures using products of a single company. This nomenclature is often based on the name of vitrification device, for instance Cryotop method which is introduced by Kitazato BioPharma Co. (Cobo *et al.*, 2008; 2012; Kuwayama, 2007).

Previous reports have mostly focused on recognizing the best cryopreservation method with the maximum clinical yields (Serdarogullari *et al.*, 2019; Fasano *et al.*, 2014; Wirleitner *et al.*, 2016). To the best of our knowledge, very little to no effort has been made to compare different vitrification methods in terms of final results despite the wide variety of the commercial products. It is expected that various commercial products have different pros and cons. In addition, the availability of the product for assisted reproductive technology (ART) clinic may also influence the clinical results due to the instability of the media components.

This retrospective study aimed at assessment of the post-warming survival rate and clinical outcomes of blastocyst stage embryos vitrified/warmed using the commercial products introduced by Ravan Sazeh Co. (CryoTouch method) and compare them with the products of Kitazato (Cryotop method).

MATERIALS AND METHODS

Study population

Infertile couples referred to Erfan Niayesh Hospital Tehran, Iran and Mehr Fertility Research Center, Rasht, Iran, between January 2018 and December 2020 were evaluated for eligibility to enter this retrospective study. Institutional Review Board of each center approved the study protocol based on the Ethical Principles for Medical Research.

Registered documents of each couple were reviewed and information regarding demographic characteristics, ovarian stimulation protocol, fertilization method, embryo culture technique, embryo grading system, vitrification/warming protocols, frozen embryo transfer (FET) protocol, endometrial preparation method and clinical outcomes were all extracted. A total of 50 FET cycles using blastocysts vitrified/warmed on day 5 of development were considered to enter the study after consideration of the following inclusion/exclusion criteria:

-The inclusion criteria consisted of age range between 20-38 years old, body mass index (BMI) < 30 kg/m², serum level of follicle-stimulating hormone (FSH) < 10 mIU/ml on the day 3 of menstrual cycle, having more than 5 blastocyst stage embryos produced by intracytoplasmic sperm injection (ICSI) fertilization method and undergoing their first ICSI/FET cycle with gonadotropin-releasing hormone (GnRH) antagonist protocol for ovarian stimulation.

-The exclusion criteria were presence of space-occupying lesions in the uterus, anatomical anomalies, hormonal dysfunctions, ovarian hyperstimulation syndrome (OHSS), immunological diseases and syndromes, hydrosalpinx, endometriosis, ectopic pregnancy, repeated implantation failure (RIF), miscarriage, and having less than 5 blastocyst stage embryos produced by ICSI method.

Ovarian stimulation protocol

All of the selected couples had received GnRH antagonist protocol for ovarian stimulation. The protocol was initiated by the administration of estradiol valerate (2 mg, PO, BID; Aburaihan Co., Tehran, Iran) from day 21 of the previous cycle and continued until days 2-4 of the subsequent cycle. Afterwards, recombinant FSH (150-225 IU, daily; Gonal F, Merck, Germany) was administered from day 2 or 3 of the cycle. Transvaginal ultrasonography was performed to monitor the follicular growth. The administration of GnRH antagonist (Cetrotide, Merck, Germany) was performed when at least one follicle reached the size of 13-14 mm in diameter and the administration continued until the day of ovulation induction. The final oocyte maturation and ovulation induction was conducted by injection of human chorionic gonadotropin (hCG, 10,000 IU; Chorion, IBSA, Switzerland). Finally, ovum pick-up (OPU) was performed 36 hours after hCG injection.

Cumulus cell-oocyte complex retrieval, oocyte denudation and fertilization

Culture media preparation was performed 8 hours prior to use by adding protein supplement (10% V/V of human serum albumin) and equilibration at 37°C in 6% CO₂ incubator. Cumulus cell-oocyte complexes (COCs) were retrieved from follicular fluid immediately after OPU. Then, the COCs were washed in a handling medium (HTF w/HEPES, Fertilite®, Ravan Sazeh Co., Tehran, Iran) and maintained in embryo culture medium (SingleCulture Medium, Fertilite®, Ravan Sazeh Co., Tehran, Iran) until denudation. Oocyte denudation was performed using both enzymatic (Hyaluronidase, Fertilite®, Ravan Sazeh Co., Tehran, Iran) and mechanical methods. ICSI was conducted on all metaphase II oocytes 3-4 hours after OPU. Afterwards, the injected

oocytes were cultured in 30-50 µL of first step sequential embryo culture medium (G1 Medium, Fertilite®, Ravan Sazeh Co., Tehran, Iran) under mineral oil (RS Medical, Ravan Sazeh Co., Tehran, Iran) overlay. After 3 days, the embryos were transferred to fresh second step sequential embryo culture medium (G2 Medium, Fertilite®, Ravan Sazeh Co., Tehran, Iran) and cultured until blastocyst stage. The resulting blastocysts were evaluated morphologically and top-quality blastocysts were selected for vitrification.

Blastocyst quality assessment

Blastocyst grading was performed according to the Gardner grading system (Wirleitner *et al.*, 2016) as following: expansion score 0 = no cavity, score 1 = blastocoel cavity less than half volume of the embryo, score 2 = blastocoel cavity more than half volume of the embryo, score 3 = blastocoel cavity completely filling the embryo, score 4 = blastocoel cavity larger than the embryo and thinning zona, score 5 = hatching blastocyst; for inner cell mass (ICM), Grade A = formed by many tightly packed cells, Grade B = several loosely packed cells, Grade C = few cells; for trophectoderm (TE), Grade A = many cells forming a cohesive layer, Grade B = few cells and loose layer, Grade C = very few large cells. Top-quality blastocysts were defined as blastocysts with expansion grades 4-5 or 2-3, and ICM and TE with AA, AB or BA classifications.

Vitrification and warming procedures

Top-quality blastocysts were selected for the vitrification using two commercial methods and the procedure was conducted according to the manufacturer's protocol, CryoTouch method (RS Medical, Ravan Sazeh Co., Tehran, Iran) or Cryotop method (Kitazato BioPharma Co., Shizuoka, Japan). Both protocols were similar with little difference. In brief, the equilibration was conducted in equilibration solution for 12-15 minutes in both CryoTouch and Cryotop methods at room temperature. Then, the blastocysts were placed in vitrification solution, then aspirated with minimum volume of the vitrification solution and finally placed onto the tip of CryoTouch® or Cryotop® vitrification devices within 50-60 sec. The loaded devices were immediately put vertically into liquid nitrogen, then placed in goblet and stored in liquid nitrogen tank.

In the morning of blastocyst transfer day, the embryos were warmed in the corresponding commercial media using the protocol provided by each manufacturer, CryoTouch and Cryotop methods. Briefly, the vitrification device was rapidly removed from liquid nitrogen and immersed in pre-warmed warming solution. Then, the blastocysts were detected and immediately transferred to another droplet of warming solution and incubated for 1 minute. Subsequently, the blastocysts were incubated in the dilution solution for 3 minutes. Next, the blastocysts were incubated in the washing solution for 5 minutes. Eventually, the blastocysts were maintained in embryo culture medium (G2 Medium, Fertilite®, Ravan Sazeh Co., Tehran, Iran) until FET.

Endometrial preparation and embryo transfer

Hormone replacement therapy (HRT) was conducted for endometrial preparation. First, estradiol valerate (6 mg/day, PO; Aburaihan Co., Tehran, Iran) was initiated from day 2-3 of the menstrual cycle and continued up to 8 mg/day until the endometrial thickness reached 8 millimeters. Then, progesterone (400 mg, suppository, BID; Cyclogest, Actavis, England, UK) was administered when the endometrial thickness was upper 8 millimeters. In the presence of positive result for β-hCG test, the estradiol and progesterone administrations were continued until weeks 6 and 12 of gestation, respectively.

Embryo transfer (ET) was performed by an expert gynecologist using an embryo transfer catheter (Guardia™ Access, Cook, USA) under the ultrasonography guide according to the guideline provided by American Society

for Reproductive Medicine (ASRM). Single top-quality vitrified/warmed blastocyst was selected for each ET cycle in each couple.

Outcome assessment

The post-warming blastocyst survival rate was calculated as the percentage of survived blastocysts after warming. Clinical pregnancy rate was calculated from the number of observed gestational sacs by ultrasonography per blastocyst transfer. Live birth rate was calculated from the number of live births per the blastocyst transfer.

Statistical analysis

All the obtained data were analyzed using GraphPad Prism software (V8, US). Comparisons of the means were conducted by Student's *t* test. The *p*-value was considered significant at < 0.05 level. Data are represented as mean \pm standard deviation (SD).

RESULTS

A total of 50 ICSI/FET cycles were evaluated during the study. Among these cycles, 27 vitrification/warming cycles were performed using CryoTouch method and 23 vitrification/warming cycles were conducted by Cryotop method. As shown in Table 1, there were no significant difference between the two groups in mean age, BMI, duration of infertility, type of infertility (primary or secondary), serum level of day 3 FSH, serum level of Anti-Mullerian hormone (AMH), and sperm count of spouse.

Table 2 represents the characteristics of the studied patients during ovarian stimulation including total dose and duration of gonadotropin, serum levels of luteinizing hormone (LH), Estradiol (E2) and Progesterone (P) on trigger

day, number of retrieved COCs, and percent of top-quality blastocysts resulted from ICSI. According to the results, there were no significant difference in these characteristics between the patients who were undergone ICSI/FET cycles using the CryoTouch or Cryotop methods.

A total of 56 blastocysts were warmed and 50 ET cycles were conducted using single top-quality blastocyst. Among 56 blastocysts, 30 were vitrified/warmed using CryoTouch method and 26 were vitrified/warmed using Cryotop method.

Post-warming survival rates of blastocysts were 94.44% for CryoTouch method (95% CI 88.11-100.8) and 93.48% for Cryotop method (95% CI 86.03-100.9), with no significant difference between the groups (*p*-value=0.8381) (Table 3).

Clinical pregnancy rates were 44.44% for CryoTouch method (95% CI 24.41-64.48) and 47.83% for Cryotop method (95% CI 25.74-69.91), with no significant difference between the groups (*p*-value=0.8157) (Table 3).

Live birth rates were 29.63% for CryoTouch method (95% CI 11.22- 48.04) and 34.78% for Cryotop method (95% CI 13.72-55.84), with no significant difference between the groups (*p*-value=0.7041) (Table 3).

DISCUSSION

Herein, the results revealed no difference in clinical outcomes of blastocysts that were vitrified/warmed by RS CryoTouch or Cryotop methods. Indeed, post-warming survival rate, clinical pregnancy and live birth were comparable in both commercial products.

Embryo development to the blastocyst stage requires successful genomic activation and passing critical developmental steps, therefore when an embryo reaches

Table 1. Demographic features of the studied groups.

| Characteristics | Groups | | |
|---------------------------------|--|--|-----------------|
| | Cryotop method | CryoTouch method | <i>p</i> -value |
| Number of ICSI/FET cycles | 23 | 27 | - |
| Age (years) | 33.04 \pm 4.61 | 31.15 \pm 5.41 | 0.1933 |
| BMI | 26.00 \pm 1.97 | 26.89 \pm 2.27 | 0.1505 |
| Duration of infertility (years) | 5.82 \pm 2.34 | 4.33 \pm 2.86 | 0.0523 |
| Type of infertility (primary) | 78.26% | 66.67% | 0.3731 |
| Type of infertility (secondary) | 21.74% | 33.33% | 0.3731 |
| FSH (Day 3) (mIU/ml) | 5.37 \pm 1.32 | 4.66 \pm 1.55 | 0.0895 |
| AMH (ng/ml) | 3.59 \pm 1.56 | 3.82 \pm 2.42 | 0.6916 |
| Sperm (count/ml) | (58 \pm 50) \times 10 ⁶ | (75 \pm 53) \times 10 ⁶ | 0.2393 |

AMH: Anti-Mullerian hormone; BMI: Body mass index; FSH: Follicle stimulating hormone; NS: Non-significant.

Table 2. Ovarian stimulation characteristics and outcomes in the studied groups.

| Characteristics | Groups | | |
|--|------------------|-------------------|-----------------|
| | Cryotop method | CryoTouch Method | <i>p</i> -value |
| Total dose of administrated gonadotropin (IU) | 1843 \pm 321 | 1907.57 \pm 445 | 0.5635 |
| Duration of gonadotropin administration (days) | 10.74 \pm 1.28 | 10.81 \pm 0.92 | 0.8101 |
| LH (IU/mL) on day of trigger | 5.13 \pm 7.64 | 4.17 \pm 0.86 | 0.5208 |
| E2 (pg/mL) on day of trigger | 2923 \pm 704 | 2748 \pm 590 | 0.3445 |
| P (IU/mL) on day of trigger | 0.72 \pm 0.31 | 0.89 \pm 0.29 | 0.0548 |
| Number of retrieved COCs | 14.6 \pm 4.3 | 11.2 \pm 5.2 | 0.1898 |
| Number of top-quality blastocysts | 2.60 \pm 1.07 | 3.40 \pm 1.86 | 0.0764 |

| Table 3. Post-warming survival rate and clinical outcomes of vitrified/warmed blastocysts in the studied groups. | | | |
|---|------------------------------|--------------------------------|----------------|
| Variables | Groups | | |
| | Cryotop method (n=23) | CryoTouch Method (n=27) | p-value |
| Number of warmed blastocysts | 26 | 30 | - |
| Blastocyst post-warming survival rate | 93.48% | 94.44% | 0.8381 |
| Clinical pregnancy rate | 47.83% | 44.44% | 0.8157 |
| Live birth rate | 34.78% | 29.63% | 0.7041 |

this stage it is considered to be highly competent (Youssry *et al.*, 2008). In the past decade, single blastocyst transfer has become a common procedure in ART clinics worldwide because of more success rates in clinical results (Wang *et al.*, 2021). This necessitates a reliable cryopreservation method for saving the remaining blastocysts which are to be used in the following transfer cycles.

There are multiple variables which impact the survival rates of the embryos during vitrification/warming processes. The most important factors that influence the effectiveness of the procedure include the concentration of cryoprotectants in vitrification/warming solutions, duration of embryo exposure to these cryoprotectants, temperature in which the procedure is being performed and the type of vitrification device which influences the cooling rate and the size of vapor coat (Al-Azawi *et al.*, 2013). An ideal strategy for improving the efficiency of vitrification is to increase the speed of thermal conduction and decrease the concentration of cryoprotectants. As most of the cryoprotectants included in commercial vitrification solutions have some degrees of cellular toxicity, it is always desired to formulate a solution containing a mixture of cryoprotectants with the lowest toxicity for embryonic cells (Mori *et al.*, 2015). As claimed by the manufacturers, both CryoTouch and Cryotop methods' vitrification media contain similar cryoprotectants with comparable concentrations (Mori *et al.*, 2015; Mori & Kuwayama, 2009). Therefore, the observed similarity in blastocyst survival rate after vitrification/warming using these commercial methods may be the consequence of this similarity of components and indicates their equality in efficiency for human blastocyst vitrification.

It is assumed that prolonged exposure to cryoprotectants may increase the possibility of toxicity (Abdel-Hafez *et al.*, 2010; Loutradi *et al.*, 2008). It is always desirable to enhance the speed of the vitrification procedure in order to avoid possible toxicities. In addition, high speed will reduce the likelihood of osmotic injury as the embryos are held in solutions without mineral oil overlay during vitrification/warming procedures (Fasano *et al.*, 2014). The incubation time in the equilibration solution was equal in both CryoTouch and Cryotop methods.

Different protocols have been presented for vitrification so far. Due to the differences in basic characteristic of the studied population as well as technical differences in ART clinics, the reported clinical outcomes are not always homogeneous (Wirleitner *et al.*, 2016; De Vos *et al.*, 2016; Debrock *et al.*, 2015). In a study conducted on 6019 embryos undergoing vitrification/warming using Cryotop method in different developmental stages, it was concluded that nearly 95% of day 5 blastocysts had 100% of intact cells (Cobo *et al.*, 2012). A survival rate of 95% was also reported by Ferreux *et al.* (2018) in a retrospective cohort follow-up study conducted on 1347 frozen-thawed blastocysts. In line with these studies, we showed ~94% blastocyst survival rate using both CryoTouch and Cryotop

methods. The efficiency of Cryotop method for vitrification/warming of human embryo and oocyte is demonstrated in previous studies (Cobo *et al.*, 2012; Braga *et al.*, 2016; Liu *et al.*, 2020; La Marca *et al.*, 2019). However, there is no published data, to the best of our knowledge, regarding the efficiency of CryoTouch method for blastocyst cryopreservation despite its routine use in infertility clinics of Iran.

Over the past decade, the number of reported live births obtained from vitrified/warmed blastocysts has substantially increased (Al-Azawi *et al.*, 2013; Wang *et al.*, 2021). However, the reported data for clinical pregnancy and live births are not homogenous. Cobo *et al.* (2012) reported pregnancy rate of 43%, and live birth rate of 40.6% with vitrified/warmed blastocysts. Kaye *et al.* (2017) reported a clinical pregnancy rate of nearly 63% after single vitrified/warmed blastocysts transfer. Ferreux *et al.* (2018) reported a clinical pregnancy rate of 43.2% and live birth rate of 29.6% resulting from vitrified/warmed blastocysts. Our results for clinical pregnancy and live birth rates were comparable with these mentioned studies. Furthermore, we showed no difference in clinical pregnancy and live birth rates between blastocysts vitrified/warmed using CryoTouch and Cryotop methods.

Although successful pregnancies are reachable following the transfer of embryos with less than 50% of the survived cells post-warming (Veiga *et al.*, 1987), pregnancy rates are higher when all the embryonic cells survive during vitrification/warming procedure. Indeed, if embryos survive the process with all its cells, the pregnancy rate will be comparable with that of fresh ET cycles (Al-Azawi *et al.*, 2013). Human embryo at blastocyst stage has different physiological requirements than cleavage stage embryo. These differences influence the chance of survival after being subjected to non-physiological conditions like vitrification (Zeng *et al.*, 2018). A major factor that has effect on survival rate of the blastocyst is the blastocoele cavity. As expected, ice crystal formation is directly proportional to the volume of the cavity (Ochota *et al.*, 2017). Recently, artificial shrinkage of the blastocoele cavity is proposed to enhance the post-warming survival rate of expanded blastocysts (Darwish & Magdi, 2016; Kovačić *et al.*, 2018). Overall, it is essential to consider these differences of blastocyst and cleavage stage embryos in efforts to establish a simple and reliable procedure to optimize blastocyst vitrification in order to have the best possible clinical outcome.

CONCLUSIONS

In conclusion, similar outcomes were observed in blastocysts vitrified/warmed using CryoTouch and Cryotop commercial methods. These results provide strong evidence regarding the comparable efficiency of vitrification/warming media and device produced by Ravan Sazeh Co. (CryoTouch method) and Kitazato (Cryotop method). This is hopeful for infertility clinics in Iran that are routinely using CryoTouch method because of its availability, low

cost, and prolonged expiration date as it is domestically manufactured in Iran.

Abbreviations

AMH: Anti-Mullerian hormone; ART: Assisted reproductive technology; ASRM: American Society for Reproductive Medicine; BMI: Body mass index; COCs: Cumulus cell-oocyte complexes; E2: Estradiol; ET: Embryo transfer; FET: Frozen embryo transfer; FSH: Follicle-stimulating hormone; GnRH: Gonadotropin-releasing hormone; hCG: Human chorionic gonadotropin; HRT: Hormone replacement therapy; ICSI: Intracytoplasmic sperm injection; IVF: In vitro fertilization; LH: Luteinizing hormone; OHSS: Ovarian hyperstimulation syndrome; OPU: Ovum pick-up; P: Progesterone; RIF: Repeated implantation failure.

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Authors' contributions

Somayeh Keshavarzi, Azadeh Dokht Eftekhari, Marzieh Mehrafza, Robabeh Taheripناه: conceptualization; methodology; Hajar Vahabzadeh, Masoumeh Asgharnia, Hossein Hosseinirad, Shahrokh Paktinat: collected, analyzed, and interpreted the patients' data; Sahar Esfand-yari, Alaleh Ghazifard: drafting the manuscript; Hossein Hosseinirad, Shahrokh Paktinat: revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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CONFLICT OF INTERESTS

The authors declare that there are no competing interests related to the subject matter or materials discussed in this article.

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REFERENCES

AbdelHafez FF, Desai N, Abou-Setta AM, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. *Reprod Biomed Online*. 2010;20:209-22. PMID: 20113959 DOI: 10.1016/j.rbmo.2009.11.013

Al-Azawi T, Tavukcuoglu S, Khaki AA, Al Hasani S. Cryopreservation of human oocytes, zygotes, embryos and blastocysts: A comparison study between slow freezing and ultra rapid (vitrification) methods. *Middle East Fertil Soc J*. 2013;18:223-32. DOI: 10.1016/j.mefs.2012.10.008

Braga DP, Setti AS, Figueira RC, Azevedo Mde C, Iaconelli A Jr, Lo Turco EG, Borges E Jr. Freeze-all, oocyte vitrification, or fresh embryo transfer? Lessons from an egg-sharing donation program. *Fertil Steril*. 2016;106:615-22. PMID: 27262501 DOI: 10.1016/j.fertnstert.2016.05.004

Cobo A, Bellver J, Domingo J, Pérez S, Crespo J, Pellicer A, Remohí J. New options in assisted reproduction technology: the Cryotop method of oocyte vitrification. *Reprod Biomed Online*. 2008;17:68-72. PMID: 18616893 DOI: 10.1016/S1472-6483(10)60295-7

Cobo A, de los Santos MJ, Castellò D, Gámiz P, Campos P, Remohí J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertil Steril*. 2012;98:1138-46.e1. PMID: 22862909 DOI: 10.1016/j.fertnstert.2012.07.1107

Debrock S, Peeraer K, Fernandez Gallardo E, De Neubourg D, Spiessens C, D'Hooghe TM. Vitrification of cleavage stage day 3 embryos results in higher live birth rates than conventional slow freezing: a RCT. *Hum Reprod*. 2015;30:1820-30. PMID: 26089301 DOI: 10.1093/humrep/dev134

Darwish E, Magdi Y. Artificial shrinkage of blastocoel using a laser pulse prior to vitrification improves clinical outcome. *J Assist Reprod Genet*. 2016;33:467-71. PMID: 26843389 DOI: 10.1007/s10815-016-0662-z

De Vos A, Van Landuyt L, Santos-Ribeiro S, Camus M, Van de Velde H, Tournaye H, Verheyen G. Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. *Hum Reprod*. 2016;31:2442-9. PMID: 27619768 DOI: 10.1093/humrep/dew219

Fasano G, Fontenelle N, Vannin AS, Biramane J, Devreker F, Englert Y, Delbaere A. A randomized controlled trial comparing two vitrification methods versus slow-freezing for cryopreservation of human cleavage stage embryos. *J Assist Reprod Genet*. 2014;31:241-7. PMID: 24317854 DOI: 10.1007/s10815-013-0145-4

Ferreux L, Bourdon M, Sallem A, Santulli P, Barraud-Lange V, Le Foll N, Maignien C, Chapron C, de Ziegler D, Wolf JP, Pocate-Cheriet K. Live birth rate following frozen-thawed blastocyst transfer is higher with blastocysts expanded on Day 5 than on Day 6. *Hum Reprod*. 2018;33:390-8. PMID: 29394365 DOI: 10.1093/humrep/dey004

Kaye L, Will EA, Bartolucci A, Nulsen J, Benadiva C, Engmann L. Pregnancy rates for single embryo transfer (SET) of day 5 and day 6 blastocysts after cryopreservation by vitrification and slow freeze. *J Assist Reprod Genet*. 2017;34:913-9. PMID: 28500451 DOI: 10.1007/s10815-017-0940-4

- Kovačić B, Taborin M, Vlaisavljević V. Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming - a prospective observational study using time-lapse microscopy. *Reprod Biomed Online*. 2018;36:121-9. PMID: 29212605 DOI: 10.1016/j.rbmo.2017.10.111
- Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology*. 2007;67:73-80. PMID: 17055564 DOI: 10.1016/j.theriogenology.2006.09.014
- La Marca A, Dal Canto M, Buccheri M, Valerio M, Mignini Renzini M, Rodriguez A, Vassena R. A novel transnational fresh oocyte donation (TOD) program based on transport of frozen sperm and embryos. *Hum Reprod*. 2019;34:285-90. PMID: 30520998 DOI: 10.1093/humrep/dey331
- Liu H, Zhang J, Wang B, Kuang Y. Effect of endometrial thickness on ectopic pregnancy in frozen embryo transfer cycles: an analysis including 17,244 pregnancy cycles. *Fertil Steril*. 2020;113:131-9. PMID: 31727414 DOI: 10.1016/j.fertnstert.2019.09.003
- Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril*. 2008;90:186-93. PMID: 17980870 DOI: 10.1016/j.fertnstert.2007.06.010
- Mori C, Kuwayama M. 167. The role of the cryoprotective effect of SSS on bovine oocytes during vitrification. *Cryobiology*. 2009;59:416-7. DOI: 10.1016/j.cryobiol.2009.10.181
- Mori C, Yabuuchi A, Ezoe K, Murata N, Takayama Y, Okimura T, Uchiyama K, Takakura K, Abe H, Wada K, Okuno T, Kobayashi T, Kato K. Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solutions for embryo vitrification in human assisted reproductive technologies. *Reprod Biomed Online*. 2015;30:613-21. PMID: 25892497 DOI: 10.1016/j.rbmo.2015.02.004
- Ochota M, Wojtasik B, Nizański W. Survival rate after vitrification of various stages of cat embryos and blastocyst with and without artificially collapsed blastocoel cavity. *Reprod Domest Anim*. 2017;52:281-7. PMID: 27859772 DOI: 10.1111/rda.12826
- Serdarogullari M, Coban O, Boynukalin FK, Bilgin EM, Findikli N, Bahceci M. Successful application of a single warming protocol for embryos cryopreserved by either slow freezing or vitrification techniques. *Syst Biol Reprod Med*. 2019;65:12-9. PMID: 29952660 DOI: 10.1080/19396368.2018.1487477
- Veiga A, Calderon G, Barri PN, Coroleu B. Pregnancy after the replacement of a frozen-thawed embryo with less than 50% intact blastomeres. *Hum Reprod*. 1987;2:321-3. PMID: 3624431 DOI: 10.1093/oxfordjournals.humrep.a136542
- Wang N, Zhao X, Ma M, Zhu Q, Wang Y. Effect of Day 3 and Day 5/6 Embryo Quality on the Reproductive Outcomes in the Single Vitrified Embryo Transfer Cycles. *Front Endocrinol (Lausanne)*. 2021;12:641623. PMID: 34046010 DOI: 10.3389/fendo.2021.641623
- Wirleitner B, Schuff M, Stecher A, Murtinger M, Vanderzwalmen P. Pregnancy and birth outcomes following fresh or vitrified embryo transfer according to blastocyst morphology and expansion stage, and culturing strategy for delayed development. *Hum Reprod*. 2016;31:1685-95. PMID: 27270972 DOI: 10.1093/humrep/dew127
- Youssry M, Ozmen B, Zohni K, Diedrich K, Al-Hasani S. Current aspects of blastocyst cryopreservation. *Reprod Biomed Online*. 2008;16:311-20. PMID: 18284893 DOI: 10.1016/S1472-6483(10)60591-3
- Zeng M, Su S, Li L. Comparison of pregnancy outcomes after vitrification at the cleavage and blastocyst stage: a meta-analysis. *J Assist Reprod Genet*. 2018;35:127-34. PMID: 28940130 DOI: 10.1007/s10815-017-1040-1