Back to life from frozen state

A Kaluarachchi[a]

History of In Vitro Fertilization (IVF)

Interest in In vitro fertilization (IVF) started way back in 1878 when the German Scientist W. Schrenk attempted to fertilize rabbit eggs in the laboratory. In 1934 Gregory Pincus was able to fertilize rabbit eggs in the laboratory. John Rock of Harvard University in 1944 mixed human eggs from cadavers with sperms and observed fertilization. In 1953, Dr Landrum Shettles fertilized human eggs to grow up to a solid mass of cells. Dr M. C. Chang in 1960 performed In Vitro Fertilization and Embryo Transfer (IVF-ET) on a female rabbit and achieved a pregnancy. In 1961 Italian Doctor Daniele Petrucci completed a successful IVF using human gametes resulting in an embryo which was destroyed after 29 days due to tissue malformation[1].

The first pregnancy achieved following IVF of a human oocyte was reported by the Monash IVF team in the Lancet in 1973[2]. It only lasted few days and would today be called a biochemical pregnancy. Robert Edwards and Patrik Steptoe achieved the first IVF-ET pregnancy in 1976 but it was an ectopic pregnancy. In July, 1978 the first IVF baby Louise Brown was born after many years of research and several attempts of unsuccessful procedures by Robert Edwards and Patrik Steptoe[1]. Louise Brown celebrated her 40th birthday in 2018. She is now a mother of two sons. During this period eggs were aspirated during natural cycles. Timing of ovum retrieval was difficult and very often patients were admitted to hospital for days and ovum retrieval done at any time of the day depending on follicular maturation. Ovum aspiration was done through the laparoscope.

A very significant milestone in IVF was the development of the Intra Cytoplasmic Sperm Injection (ICSI) of a single sperm in Brussels in 1992[3]. This has enabled men with minimal sperm production to achieve pregnancies, sometimes in conjunction with sperm recovery using a testicular fine needle aspiration or open testicular biopsy. Some men even with Kline-felter syndrome have occasionally been successful. Subsequently there have been many advances in the field including frozen embryo transfer, donor programs and in vitro maturation of oocytes etc. Thus, IVF has become the final solution for most fertility problems including tubal disease, male factor infertility, unexplained subfertility, endometriosis, advanced maternal age, and anovulation not responding to ovulation induction.

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[a] *Professor in Obstetrics and Gynaecology, Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Colombo, Sri Lanka.*

Correspondence: AK, e-mail: <athula_kal@yahoo.com>

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https://orcid.org/0000-0003-0889-7716

The steps of Assisted Reproductive Techniques (ART)

The key steps include:

- Pituitary down regulation
- Ovarian hyper stimulation
- Egg collection by aspiration of follicles
- Collection of semen or sperm recovery
- Fertilization by conventional IVF or ICSI
- Transfer of resulting embryos to the uterus
- Luteal support (administration of hormones to aid implantation of embryos)

The process of ART includes initial pituitary down regulation which means that you remove the control of the ovaries by the pituitary with administration of GnRH agonists or antagonists followed by ovarian hyperstimulation with gonadotropin injections. Individualized protocols are used depending on patient characteristics. The follicle response is monitored by transvaginal ultrasound scans.

On successful ovarian hyperstimulation the egg retrieval is performed under local anesthesia commonly using a paracervical block. Transvaginal approach is used with the single or double channel needle under the ultrasound guidance. Follicular fluid is aspirated in to test tubes which are kept at 37 degrees Celsius. The fluid collected is transferred to the laboratory immediately for screening for oocytes. In the laboratory follicular fluid is screened for oocytes in a petri dish. All the oocytes collected are washed and incubated for up to 6 hours prior to insemination. On the same day a sperm sample is processed to retrieve good quality spermatozoa for insemination.

Conventional IVF is where the egg is incubated with 20000 - 30000 sperms. However only a single sperm will enter the oocyte. This process allows natural selection of a sperm for fertilization. When the sperm count is very low, insemination is performed by injecting a single sperm in to the egg. Intra Cytoplasmic Sperm Injection (ICSI) is performed by using a micromanipulator. Once inseminated, observations are made to identify fertilization. It is important to be certain that only 2 pronuclei are present which confirms that only one sperm has entered the egg so that the chromosome number is 46. The embryo transfer is performed on Day 3 or day 5 at the blastocyst stage.

The reasons why an embryo may not be transferred in the fresh cycle include:

- Risk of ovarian hyperstimulation
- The storage of surplus embryos of good quality
- Postponing embryo transfer in special cases such as poor endometrium
- The preservation of reproductive potential of a woman prior to chemotherapy, radiotherapy or oophorectomy.

Successful cryopreservation of mammalian embryos yielding live offspring

The key reports in history of cryopreservation include[4]

Species	First report
Mouse	Whittingham et al. 1972
Cattle	Willmut & Rowson 1973
Rabbit	Bank & Maurer 1974
Sheep	Willadsen et al. 1974
Rat	Whittingham 1975
Goat	Bilton & Moore 1976
Horse	Yamamoto et al. 1982
Human	Trunson & Mohr 1983
Eland	Kramer et al. 1983
Baboon	Pope et al. 1984
Marmoset	Summers et al. 1986
Macaque Monkey	Balmaceda et al. 1987
Cat	Dresser et al. 1988

The first successful pregnancy with cryopreservation of a human embryo was reported from Melbourne, Australia in 1983 and this was published in the journal Nature[5]. The embryo was "frozen" for two months before transfer into the mother's uterus[5]. The pregnancy was terminated at 24 weeks due to sepsis.

Techniques have been developed to store embryos in liquid nitrogen (referred to as cryopreservation) for an indefinite period of time without significant compromise of their quality. The fundamental objectives for successful cryopreservation of cells in liquid nitrogen at -196°C are as follows: 1) arresting the metabolism

reversibly 2) maintaining structural and genetic integrity 3) achieving acceptable survival rates after thawing 4) maintaining of developmental competence post thaw 5) the technique has to be reliable and repeatable[6].

Cryopreservation at low temperature slows or totally prevents unwanted physical and chemical change. The major disadvantage of using low temperature cryostorage is that it can lead to the crystallization of water that may injure the cells that are being preserved. All methods and protocols for cryopreservation should be such that ice crystals formation and growth inside the cells or tissues must either be eliminated or massively suppressed. The slow-cooling or rapid-cooling protocols (Vitrification) both satisfy the fundamental cryo-biological principles for reduction of damage by ice crystal formation during cooling and warming.

Both protocols of cryopreservation of biological material include six principal steps: 1) initial exposure to the cryoprotectant (intracellular water has to be removed by gradual dehydration, 2) cooling (slow/rapid) to subzero temperatures (-196°C), 3) storage at low temperature, 4) thawing/warming by gradual rehydration, 5) dilution and removal of the cryoprotectant agents and replacement of the cellular and intracellular fluid at precise rate and, 6) recovery and return to a physiological environment[6].

Cryoprotectant agents are essential for the cryopreservation of cells. Basically two groups of cryoprotectants exist: 1) permeating *(glycerol, ethylene glycol, dimethyl)* and 2) non-permeating *(saccharides, protein, polymers)* agents.

The downside to the slow freezing approach is the time to complete such freezing procedures for human embryos, which can range from 1.5 to 5 hrs. This is due to the fact that the slow rate of cooling attempts to maintain a very delicate balance between multiple factors that may result in cellular damage by ice crystallization and osmotic toxicity. Although the results achieved by slow freezing in many cases seem quite successful[7,8], ice crystal formation still renders traditional slow freezing programs generally less consistent in their clinical outcomes. Traditionally slow-freeze embryo cryopreservation has been a positive contributor to cumulative patient pregnancy rates, but ultimately the limitations of current slow freezing methods in ART have become more evident when compared to vitrification based cryostorage.

Vitrification is one of the more exciting developments in ART in recent years that attempts to avoid ice formation altogether during the cooling process by establishing a glassy or vitreous state rather than an ice crystalline state. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in combination with high concentrations of cryoprotectants are used. In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature.

During vitrification, by using a cooling rate in the range of 2,500 to 30,000°C/min or greater, water is transformed directly from the liquid phase to a glassy vitrified state. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice-crystallization but extreme elevation in viscosity during cooling[9,10]. Vitrification of the aqueous solution inside cells can be achieved by increasing the speed of temperature change, and by increasing the concentration of the cryoprotectant used[9,10].

In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported[11,12]. Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice-crystallization using vitrification[13]. Over 95% survival rate of frozen embryos are reported at present[6].

Study on factors affecting the outcome of frozen-thawed embryo transfer

Introduction

Frozen-thawed embryo transfer (FET) has become an essential part of IVF/ICSI treatment, increasing the cumulative pregnancy rate (PR) after ovum retrieval. Apart from increasing the cumulative pregnancy rate cryopreservation comes with the added advantages of lowering the risk of multiple pregnancy and ovarian hyperstimulation syndrome. This provides the chance to have an additional pregnancy without ovarian stimulation and an opportunity for embryo donation.

Various factors are known to affect the outcome of FET. Among them are clinical factors, such as female age at embryo freezing and embryological factors such as embryo quality[13]. A pregnancy in the fresh IVF/ ICSI cycle has been shown to be associated with an improved PR in subsequent FET[14,15]. Embryos of good quality before freezing have a better cryopreservation survival rate and are associated with a higher pregnancy rate[14]. The outcome of treatments is also improved if thawed embryos resume cleaving[16], but cryopreservation associated damage of embryos worsens the chance of a pregnancy[17].

With the continued development of scientific innovations in the assisted reproduction it is imperative to be knowledgeable about the treatment options that are offered to couples and the factors affecting the success in order to achieve the maximum benefit from the procedures.

Objective

The objective of the study was to assess the clinical and embryological factors that would affect the outcome of frozen embryo transfers in a Sri Lankan population.

Methods

All patients (N-384) who underwent IVF or intracytoplasmic sperm injection at an Assisted Reproductive Health Center (RHC) and had frozen embryo transfer from May 2011 to May 2017 were retrospectively reviewed.

Data was collected from the data base of the Assisted Reproductive Health Centre which includes the clinical and embryological factors which could affect the outcome of frozen embryo transfer cycles. Clinical data collected consists of age of the patient, type and cause for subfertility, IVF protocol and the endometrial thickness on day 12. Embryological data collected from the data base included the method of fertilization, method of freezing, storage duration of the embryo, and number of embryos transferred in each FET cycle.

Cryopreservation of embryos were carried out with the consent of the couple. Stimulation protocols included long, short and antagonist protocols. Cryopreservation was carried out on Day 3, at cell stage 6-8. Embryos were graded on early day 3 prior to cryopreservation by giving a score of 1-4 according to morphological characteristics of the embryo, cleavage rate, and presence of fragments.

Cryopreservation of embryos were carried using slow freezing technique from August 2010 to August 2015. From 2015 August freezing was done using vitrification technique.

Primary outcomes were the clinical pregnancy rate and live birth rate per cycle. Clinical pregnancy rate was defined as a conception which has a measurable beta hCG per cycle and live birth rate was defined as the number of live births per cycle. The type of subfertility was categorized as primary and secondary and the aetiologies were categorized as male factor, polycystic ovary syndrome, endometriosis, tubal and unexplained. Endometrial thickness was sub grouped in to two categories based on the cutoff of 8 mm. Cases were included in to the most severe etiological factor in order to prevent overlapping. The effect of following factors were analyzed on the live birth rate and clinical pregnancy rate. Namely age of the female partner, freezing technique, type of subfertility, aetiology of subfertility, number of embryos transferred per cycle, endometrial thickness at the time of embryo transfer and the type of IVF protocol used.

Analysis

Analysis of the data was performed by SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using chi square test and a ‘p’ value less than 0.05 was considered as statistically significant. Linear regression analysis was used to control the possible confounding variables.

Results

Total numbers of 384 frozen embryo transfer cycles were carried out during this period. Patients who underwent assisted reproduction using donor oocytes (n-14) and who did not have viable embryos after thawing (n-4) were excluded from the study. All the conventional IVF and ICSI cycles which had subsequent FET were included in the study. Accordingly, a total number of 366 FET cycles were included in the study population. However, 2 more FET cycles were excluded from analysis due to missing data in many fields. Total number of 3367 embryos were frozen and 820 embryos were transferred during this period (Table 1).

Table 1. Clinical data of subjects

Total number of FET cycles performed during the study period	384
FET done with donor oocytes	14
FET abandoned due to non viable embryos	4
FET cycles included in the analysis	364
Total number of embryos frozen	3367
Number of embryos transferred	820

The Figure 1 below shows the age distribution of the female partner. Majority of patients (49%) were between 30-35 years. The mean age of the population was 34.3 with a standard deviation of 4.1.

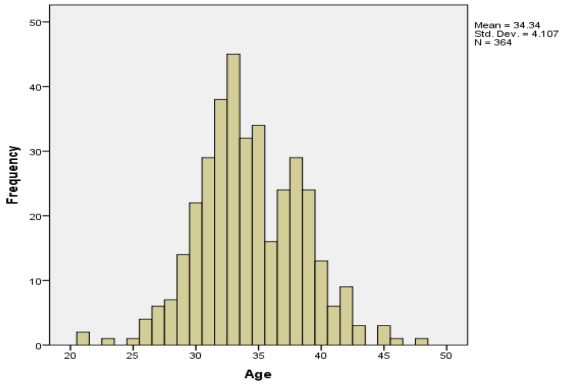

Figure 1. **Age (years) distribution of the population.**

The figure 2 below shows the aetiology of subfertility. The most common pathology seen in these women was endometriosis followed by tubal disease. Male factor infertility was seen in over 20% of couples presented.

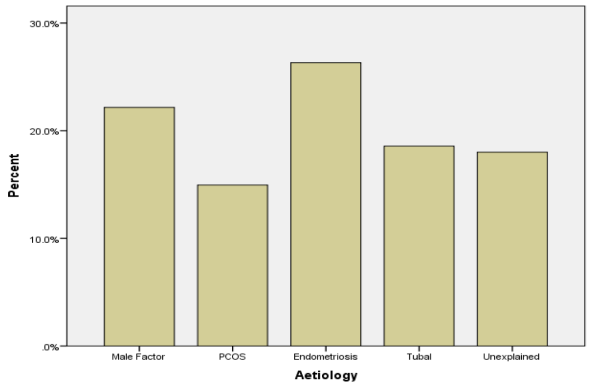

Figure 2. **Aetiology of subfertility among the population.**

The most frequent protocol used in this population was the long protocol (Figure 3) followed by the antagonist protocol.

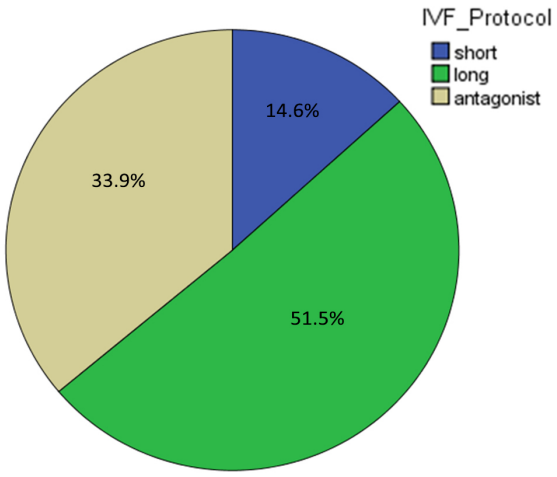

Figure 3. **Use of different IVF protocols among the population.**

The clinical pregnancy rate per cycle was 51.37% while the live birth rate per cycle was 34.06% as shown in table 2.

Women who were less than 35 years of age had a significantly higher clinical pregnancy as well as live birth rate.

The method of freezing had a significant impact on the outcome. Clinical pregnancy rate and the live birth rate was significantly higher in cases where vitrification was used as the freezing method compared to the slow freezing technique.

Whether the subfertility is primary or secondary was not significant with regard to the pregnancy outcome. When the aetiology of subfertility was compared with the pregnancy and live birth rate there was no significant difference.

The number of embryos transferred at each cycle was studied. The clinical pregnancy rate and live birth rate were significantly higher when 2 or 3 embryos were transferred compared to a single embryo transfer.

Three different protocols were used in the IVF cycles. These were Long protocol, Antagonist protocol and the Short protocol. Clinical pregnancy rates were

Table 2. Summary of clinical factors and pregnancy outcomes (N=364)

Clinical factor	Frequency of observation	Clinical pregnancy rate %	Live birth rate %	'p' value for live birth rate
Total	364	51.37%	34.06%	
Age				
<35	235	54.5	48.6	0.003
>35	129	45.7	34.3	
Freezing method				
Slow freezing	224	46.8	36.07	0.004
Vitrification	140	57.8	55.5	
Type of subfertility				
Primary subfertility	314	49.7	42.15	0.666
Secondary subfertility	50	62	52.08	
Aetiology				
Male factor	80	43.7	40.50	0.161
PCOS	67	64.8	57.69	
Endometriosis	65	52.6	50.53	
Tubal	95	49.2	35.93	
Unexplained	54	49.2	31.74	
Number of embryos transferred				
1	28	28.5	11.11	0.033
2	218	57.7	48.35	
3	116	45.6	42.85	
4	2	0	0	
Endometrial thickness				
8 mm or more	286	53.4	46.57	0.014
Less than 8 mm	76	43.4	33.33	
Protocol used				
Short	48	39.6	34.04	0.023
Long	184	48.4	41.34	
Antagonist	131	60.3	50.39	

significantly higher in the group with antagonist protocol compared to short protocol. Age presents a major confounder here as most of the time short protocol is used in patients with poor ovarian reserve and in older patients.

The live birth rates were significantly higher when the endometrial thickness was more than 8 mm, however there was no significant association between clinical pregnancy rate and endometrial thickness.

Discussion

Frozen embryo or blastocyst transfer is increasingly performed by the fertility centers with equal or better success rates compared to transfer in the fresh cycles[18,19,20]. Successful cryopreservation of embryos are essential to maximize the safety and efficacy of ovarian stimulation cycles in an IVF treatment programme. Embryo freezing also helps fertility preservation. Slow freezing and vitrification are two methods that are used. The cryopreservation method employed in ART centers has an impact on embryo quality. Most recent studies suggest that embryo freezing with vitrification is superior to slow freezing/ thawing with regard to clinical outcomes and cryosurvival rates of cleavage stage embryos and blastocysts[21,22].

In 2015, our ART center gradually shifted from slow freezing to vitrification. Therefore, data on both cryopreservation protocols were available for comparison. The decision to shift protocols was based on literature reporting higher embryo survival rate and fully intact blastomere rate in vitrified embryos.

In the current study the clinical pregnancy and live birth rates were higher when embryos were frozen with the vitrification method than when frozen using the slow freezing method. This difference was statistically significant. As in other studies the present study also confirmed that vitrification is superior to slow freezing method[21,22].

Different stimulation protocols are used in assisted reproduction. The common protocols used are long agonist protocol, Short protocol and antagonist protocol. The implantation rates were analyzed according to the different protocols used. In the present study using the antagonist protocol had a higher success rate compared to the short protocol. In the present series the short protocol was used mainly in patients with poor ovarian reserve and in older patients. Other studies suggest that the protocol used in the IVF cycle does not affect the final outcome[23].

Once the embryos are retrieved fertilization is done with conventional In Vitro Fertilisation or Intra-Cytoplasmic Sperm Injection. When the results were analysed according to the method of fertilization it did not show any statistically significant difference. This finding is similar to other studies conducted looking at factors affecting the success of FET[23].

The preparation of the endometrium is an important factor affecting the success of implantation of the embryo. The endometrial thickness is routinely assessed prior to the embryo transfer. Different studies have looked at different cut offs of endometrial thickness[24,25]. We compared the success of implantation when the endometrial thickness is less than 8mm and more than 8mm. The implantation rate was higher in the group with endometrial thickness more than 8 mm. This difference was statistically significant. This finding was comparable with the previous studies[24,25].

As in all aspects of infertility management female age was an important factor in the success of frozen embryo transfer. There was a linear decline in implantation rate with advancing age. Other studies also have shown higher success of FET in patients who are less than 35 years of age[23].

It is generally believed that the quality of the oocytes will be affected by the presence of endometriosis. It has been shown that the number of eggs retrieved are less in patients with endometriosis compared to patients without such disease. There is no conclusive data with regard to fertilization and embryo quality in patients with different stages of endometriosis[26].

In the present analysis there was no difference in success rate according to the aetiology of subfertility. This area need further studies.

Having a previous pregnancy is a positive factor for the success of in vitro fertilization. In the present analysis we did not find a significant difference in the success rate in the two groups' primary and secondary subfertility.

Pregnancies are reported following transfer of embryos after freezing for 16 years. It is important to know whether the duration of freezing affects the success

of frozen embryo transfer. The success rate was analysed according to the duration of embryo freezing. This showed a gradual decline in the implantation rate according to the duration of freezing.

The present study was important to assess the different factors affecting the success of frozen embryo transfer. It was shown the method of freezing, age of the female partner, duration of freezing and endometrial thickness are factors which could affect the final outcome of frozen embryo transfer.

References

1. Zhu, Tian, In Vitro Fertilization. Embryo Project Encyclopedia (2009-07-22). ISSN: 1940-5030 http://embryo.asu.edu/handle/10776/1665.
2. De Kretzer, D, Dennis P, Hudson B, Leeton J, Lopata A, Outch K,Wood C. Transfer of a human zygote. The Lancet, 1973; 302(7831), 728-29. doi:10.1016/s0140-6736(73)92553-1
3. Palermo G, Joris H, Devroey P, Steirteghem AC. Pregnancies after intra-cytoplasmic injection of single spermatozoon into an oocyte. The Lancet. 1992 July 4; 340(8810): 17-18.
4. Ali J, AlHarbi NH, Ali N. (2017) Chapter 1 Historical Background on Gamete and Embryo Cryopreservation. In: Nagy Z., Varghese A., Agarwal A. (eds) Cryopreservation of Mammalian Gametes and Embryos. Methods in Molecular Biology, vol 1568. Humana Press, New York, NY.
5. Trounson A, Linda M. Human pregnancy following cryopreservation thawing and transfer of an 8-cell embryo. Nature. 1983 October; 305(5936): 707-9.
6. Katkov, I. IntechOpen. [Online]. Available from: https://www.intechopen.com/books/current-frontiers-in-cryobiology [Accessed 14th January 2020].
7. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Changing the start temperature and cooling rate in a slow-freezing protocol increases human blastocyst viability. Fertil Steril 2003 79: 407-10.
8. Van den Abbeel E, Camus M, Verheyen G, Van Waesberghe L, Devroey P, Van Steirteghem A. Slow controlled-rate freezing of sequentially cultured human blastocysts: an evaluation of two freezing strategies. Hum Reprod. 2005 Oct; 20(10): 2939-45.
9. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology. 1984 Aug; 21(4): 407-26.
10. Fahy GM. Vitrification: a new approach to organ cryopreservation. In: Merryman HT (ed.), Transplantation: approaches to graft rejection, New York: Alan R Liss,1986; 305-35.
11. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: case report. Hum Reprod 1999 Dec; 4(12): 3077-9.
12. Yoon TK, Chung HM, Lim JM, Han SY, Ko JJ and Cha KY. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program [letter]. Fertil Steril 200; 74: 180-81.
13. Walker DL, Tummon IS, Hammitt DG, Session DR, Dumesic DA, Thornhill AR. (2004). Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. Reprod Biomed Online. 2004 May; 8(5): 558-68.
14. Karlstrom PO, Bergh T, Forsberg AS, Sandkvist U, Wikland M. Prognostic factors for the success rate of embryo freezing. Hum Reprod 1997; 12: 1263-66.
15. El-Toukhy T, Khalaf Y, Al-Darazi K, O'Mahony F, Wharf E, Taylor A, Braude P. Cryo-thawed embryos obtained from conception cycles have double the implantation and pregnancy potential of those from unsuccessful cycles. Hum Reprod 2003; 18: 1313-18.
16. Guerif F, Bidault R, Cadoret V, Couet ML, Lansac J, Royere D. Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. Hum Reprod 2002; 17: 1321-26.
17. Salumets A, Suikkari AM, Makinen S, Karro H, Roos A, Tuuri T. Frozen embryo transfers: implications of clinical and embryological factors on the pregnancy outcome. Hum Reprod 2006; 21: 2368-2374. Epub 2006 May 2369.
18. Wei D, Liu JY, Sun Y, Shi Y, Zhang B, Liu JQ, et al. Frozen versus fresh single blastocyst transfer in ovulatory women: a multicentre, randomized controlled trial. Lancet. 2019 Mar 30; 393(10178): 1310-1318. doi: 10.1016/S0140-6736(18)32843-5. Epub 2019 Feb 28.

19. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of Fresh versus Frozen Embryos in Ovulatory Women. N Engl J Med. 2018 Jan 11; 378(2): 126-136. doi: 10.1056/NEJMoa1705334.

20. Vuong LN, Dang VQ, Ho TM, Huynh BG, Ha DT, Pham TD, et al. IVF Transfer of Fresh or Frozen Embryos in Women without Polycystic Ovaries. N Engl J Med. 2018 Jan 11; 378(2): 137-147. doi: 10.1056/NEJMoa1703768.

21. Argyle CE, Harper JC, Davies MC. Oocyte cryopreservation: where are we now? Hum Reprod Update. 2016 Jun; 22(4): 440-9. doi: 10.1093/humupd/dmw007. Epub 2016 Mar 2.

22. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update. 2017 Mar 1; 23(2): 139-155. doi: 10.1093/humupd/dmw038.

23. Maryam Eftekhar M, Rahmani E, Pourmasumi S. Evaluation of clinical factors influencing pregnancy rate in frozen embryo transfer. Iran J Reprod Med July 2014 Vol. 12. No. 7. pp: 513-518.

24. Zhang T, Li Z, Ren X, Huang B, Zhu G, Yang W, Jin L. Endometrial thickness as a predictor of the reproductive outcomes in fresh and frozen embryo transfer cycles: A retrospective cohort study of 1512 IVF cycles with morphologically good-quality blastocyst. Medicine (Baltimore). 2018 Jan; 97(4): e9689. doi: 10.1097/MD.0000000000009689.

25. Wu Y, Gao X, Lu X, Xi J, Jiang S, Sun Y, Xi X. Endometrial thickness affects the outcome of in vitro fertilization and embryo transfer in normal responders after GnRH antagonist administration. Reprod Biol Endocrinol. 2014 Oct 9; 12: 96. doi: 10.1186/1477-7827-12-96.

26. Sanchez AM, Vanni VS, Bartiromo L, Papaleo E, Zilberberg E, Candiani M, Orvieto R, Vigano P. Is the oocyte quality affected by endometriosis? A review of the literature. J Ovarian Res. 2017 Jul 12; 10(1): 43. doi: 10.1186/s13048-017-0341-4.