Review Article

Cryopreservation- A Boon to Assisted Reproductive Technologies

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Abstract

With advancement in assisted reproduction technology, cryopreservation has now become an essential aspect of in vitro fertilization cycle. While this technology was considered to have potential benefit in Assisted Reproductive Technology (ART) earlier, in recent times it has also found its place in fertility preservation with increasing awareness and knowledge of cancer treatment and its consequences. This review gives an overview of importance of cryopreservation technology in infertility treatment and fertility preservation.

Key Words: Cryopreservation; Gametes; Embryo; Gonadal tissue

Introduction

Lazaro Spallanzani, an Italian Physiologist, for the first time in 1776 observed that spermatozoa became motionless when cooled by snow.[1] But a serendipitous discovery by Christopher Polge, paved way for the establishment of sperm cryopreservation by demonstrating the use of glycerol as a cryoprotectant preserved the motility of rooster spermatozoa.[2] The concept of semen banking in humans was first proposed by Mantegazza in 1886, for military mendesiring a progeny,by storing sperm for long periods by cryopreservation.[3] The reporting of the first human pregnancy by frozen sperm (-78°C) by Bunge and Sherman in 1953 was a milestone in infertility leading to the establishment of clinical cryobanks and evolution of cryopreservation protocols in the IVF clinic.[4]

Rapid advancement in ART has led cryopreservation to become an integral part of its treatment, by cryopreserving gametes, embryos and gonadal tissues (Fig 1). This is done by cooling of cells or whole tissues to low, sub-zero temperatures, usually in liquid nitrogen (-196°C) or in liquid nitrogen vapor (-160°C), at which temperature the cells are maintained in a state of suspended animation over a period of time due to the cessation of all metabolic processes within the cell.[5] This is feasible with the use of cryoprotective agents which are employed in cryopreservation to minimize cellular damage during the process of freeze-thawing.

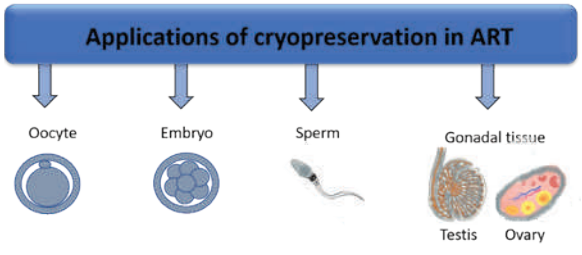

Fig 1: Current applications of cryopreservation in ART

There are two main types of cryoprotective agents insert - penetrating and no-penetrating, based on their ability to permeate through the cell membrane. Commonly used low molecular weight permeating cryoprotectants are glycerol, DMSO, propanediol and ethylene glycol. Non-penetrating agents such as trehalose, raffinose, sucrose, ficoll, plasma proteins and lipoproteins do not permeate the cells but cause dehydration by exosmosis.[6,7]

The different cryopreservation protocols used for gametes/embryos/gonadal tissues are slow freezing, rapid freezing and vitrification. The principle behind all these methods is to first expose the cells to cryoprotectants followed by cooling to the desired storage temperature, and consequently, warming of cells(thawing) along with the removal of cryoprotectants. In slow cooling procedures the cells are equilibrated with penetrating cryoprotectants by exposing them to the increasing concentrations of cryoprotectant solutions and then gradually exposed to temperatures in the decreasing order using a sophisticated automated programmable freezer. This process is usually time consuming.

The rapid cryopreservation method involves plunging of cells into liquid nitrogen after sufficient equilibration with cryoprotectants. This method is relatively simple and does not require any expensive

instruments[8] unlike slow cooling. A more commonly used method in recent times, for oocytes and embryos is vitrification which is an ultra-rapid cooling technique where the cells are exposed to high concentration of cryoprotective agents and then rapidly immersed in liquid nitrogen with a cooling rate as high as 6-10,000° C/min. This protocol is routinely utilized in cryopreserving gametes[2], zygotes[9,10], blastocysts[11,12] and gonadal tissues.[13,14]

To utilize the frozen samples for ART treatment, the cells of interest are thawed when they are taken out of liquid nitrogen and transferred to suitable culture media at 37°C. Rapid rate of thawing is preferred over slow rate as it significantly avoids the growth of intracellular ice crystal formation during this process.

The different biological material that can be cryopreserved in ART treatment and fertility preservation are:

a. Semen

Semen cryopreservation is an established ART procedure which is offered to infertile men undertaking treatment and also men with medical disorders whose treatment might compromise their fertility.Males affected with testicular cancer, Hodgkin's disease and other lymphomas, leukemia, nephrotic syndrome, diabetes, multiple sclerosis, men exposed to pesticides, radiation and mutagenic chemicals and men engaged in military operations are the potential candidates for semen cryopreservation.

Sperm banking is a common application of cryopreservation widely offered in ART, where cryopreserved sperm from anonymous healthy, screened donors can be used in treating infertile couples where the male partner has untreatable azoospermia /or total teratozoospermia. Having a large number of donor samples with different phenotypes and blood groups will help to match the recipient. More importantly, quarantine of samples can be achieved by cryopreservation.

Another application of sperm banking is to store back - up samples for infertile patients undertaking ART treatment. Men are usually required to produce a semen sample on the day of the ART procedure. This can be highly stressful to certain patients and therefore may experience difficulty in collection, due to anxiety-related erectile or ejaculatory dysfunction. This can also be beneficial to patients who might not be present on the day of the procedure due to unscheduled traveling. Storage of sperm as a precautionary measure prior to the scheduled procedure can help to alleviate the emotional stress on such patients and also ensure that treatment can proceed as planned. The common indications for sperm cryopreservation are listed in Table 1.

Indication	Application of cryopreservation
Back-up samples	For patients with difficulty in collection or unavailable on the day of procedure, this option helps to reduce the emotional burden on patients
Donor sperm banking	For heterologous insemination, semen samples can be obtained from donors, as per ICMR guidelines and cryopreserved.
Fertility preservation for cancer patients	Patients with malignant and non-malignant diseases whose treatment can impair fertility can preserve their samples before undergoing treatment. For adult males, semen cryopreservation can be offered.
Social sperm freezing/ convenience banking	For men who would like to start a family later in life can preserve their sperm while they are young
Testicular/Epididymal spermatozoa storage after TESA/TESE/PESA	Patients with obstructive and non-obstructive azoospermia can store retrieved testicular/epididymal spermatozoa samples which can be used for ICSI.

Table 1: Indications for sperm cryopreservation

The process of cryopreservation can lead to dramatic changes in intra-and extracellular environment of the sperm. According to Gao et al., at least 50% of spermatozoa lose their viability due to ultrastructural changes that happens during freeze-thaw process thereby reducing the efficacy of cryopreservation.[15,16] The ultrastructural changes include membrane and acrosome swelling, loss of acrosome and mitochondrial damage.[17-19] The damage to mitochondria and sperm tail during cryopreservation leads to poor motility in post thaw samples[16,20] which is thought to be associated with production of free radicals.[21] The high susceptibility of spermatozoa to reactive oxygen species (ROS) is due to the high PUFA content in their membrane and the limited repair ability.[22-25]

b. Oocyte

Oocyte cryopreservation gives flexibility to ART treatment by allowing the possibility of cryopreserving the oocytes in certain scenarios such as ovarian hyper stimulation syndrome (OHSS) or inability of the male partner to produce viable sperm sample on the day of procedure or for social egg freezing. Oocyte freezing is also offered as a fertility preservation procedure for cancer patients who are at risk of premature ovarian failure due to chemotherapy and/or radiotherapy.[26,27] Another important application of oocyte cryopreservation is in oocyte donation programmes where young fertile women are recruited according to ICMR guidelines[28], undergo controlled ovarian stimulation and oocytes are collected. The obtained oocytes can be cryopreserved and thawed later for indicated patients when required. The indications for oocyte cryopreservation are listed in Table 2.

The human metaphase II oocytes are vulnerable to freeze thaw procedure due to its ultrastructure, large nuclear cytoplasmic ratio, presence of meiotic spindle and sensitivity of zona pellucida.[29] The most common cryoinjuries are damage to the spindle apparatus[30,31],

Indication	Application of cryopreservation
Donor oocyte banking	Oocyte donors can be recruited as per ICMR guidelines. Their oocytes are retrieved and cryopreserved which can be used when needed for indicated after appropriate matching.
Fertility preservation for cancer patients	For women in the reproducible age and prepubertal cancer patients, oocyte cryopreservation can be offered
Social egg freezing	Women who wish to start their family late due to professional, social or financial situations, may choose to freeze their oocytes at a younger age which can later be thawed and used for ICSI.

Table 2: Indications for oocyte cryopreservation

hardening of zona pellucida[32], premature cortical granule reactions[33], chromosomal anomalies[34,35] and parthenogenesis.[36]

To overcome the above stated issues, an alternative strategy is to store immature oocytes at the germinal vesicle stage, obtained from graffian follicles. Immature oocytes are less sensitive to cryoinjury[14] due to their small size (30- 60°m), low metabolic rate, absence of zona, cortical granules and smaller amount of cryosensitive intracytoplasmic lipids.[37] Since these oocytes are at diplotene stage, the lack of spindle apparatus presents less risk of cytogenic errors in subsequent divisions. The only major hurdle for this alternative strategy is that these oocytes have to undergo invitro maturation which may lead to zona hardening.

c. Cryopreservation of gonadal tissues

(i). Testicular tissue

Cryopreservation of spermatozoa retrieved by testicular biopsies is a routinely followed ART procedure for patients with non-obstructive azoospermia which helps to overcome the financial, physical and mental strain of undergoing repeated biopsy procedure. Several studies have shown that the fertilization potential, embryo growth and delivery rate of fresh and frozen spermatozoa retrieved from testicular tissue are comparable.[38-40]

Testicular tissue cryopreservation is also offered for prepubertal cancer patients prior to chemotherapy, as a part of fertility preservation procedure, which is still experimental in nature.[41,42] The prepubertal testicular tissue is highly susceptible to cytotoxic treatments due to the presence of spermatogonia that are proliferative in nature and therefore are targeted by anti-cancer agents.[43] Hence, in these patients, testicular biopsy can be performed and the tissue can be cryopreserved by slow freezing. Fertility restoration can be done by thawing the tissue at a later time, when the patient is ready to have a family, and sperm derived by either auto-transplantation to an orthotopic or heterotopic site or even be matured in vitro.[44]

Indication	Application of cryopreservation
Testicular tissue	Male prepubertal patients can undergo testicular biopsy and cryopreserve their testicular tissue, which can be used in future to restore fertility
Ovarian tissue	Female prepubertal patients can undergo ovarian biopsy and cryopreserve their ovarian cortex, which can be used in future to restore fertility

Table 3: Indications for gonadal tissue cryopreservation

(ii). Ovarian tissue

In women who are at a risk of premature menopause due to several reasons such as recurrent or severe ovarian diseases (cysts, benign tumors, endometriomas), removal of ovary to treat endometriosis or genital cancer[45], chemotherapy or radiotherapy to treat cancer or other systemic diseases[46] and in women with chromosomal anomalies such as Turner's syndrome[45-47], ovarian tissue cryopreservation or whole ovary cryopreservation can be offered. Though this procedure is still experimental in nature, it is a promising alternative to prevent fertility loss in the above mentioned group of patients. It has been shown that ovarian tissue is more cryotolerant and regains function upon transplantation without vascular anastamosis. The indications for gonadal tissue cryopreservation are listed in Table 3.

d. Embryo cryopreservation

Embryo cryopreservation has proved to be a boon to ART as the technique helps in freezing supernumerary embryos, alleviating the stress on patients with OHSS through a frozen embryo transfer cycle, and in patients who are unable to undergo fresh ET due to inadequate endometrial development. A recent addition to the indication for embryo cryopreservation is pre-implantation genetic testing for patients with a history of genetic disease PGD - Preimplantation genetic diagnosis, or in patients with repeated IVF failures who wish to increase the chances of pregnancy PGS- Preimplantation genetic screening. The indications for embryo cryopreservation are listed in table 4.

The outcome of embryo cryopreservation is mainly influenced by the stage of development[48] and the quality of embryo at the time of freezing.[49] Embryos have been successfully cryopreserved at zygote[48], cleavage[50] and blastocyst stages[51], using various freezing protocols with either dimethylsulphoxide (DMSO)[52], 1,2-propanediol(PROH)[53] or glycerol[51] as cryoprotective agents.Currently the most widely used method for cryopreservation of embryos is vitrification using 15%DMSO, 15% EG and 0.5M sucrose.[54] However, the major concern with embryo cryopreservation is the passage of rights and disposal of embryos if the couple divorces each other or in case of death of a partner.

Indication	Applications of cryopreservation
Surplus embryo freezing	Patients who have surplus embryo after IVF/ICSI can have their embryos frozen which can used for their own future use, through an Frozen Embryo Transfer cycle
Patients with deferred ET/ FET cycles	Patients with Ovarian Hyperstimulation Syndrome and whose endometrium is not receptive for an embryo transfer can opt for embryo cryopreservation, which can be thawed and transferred when the patient is ready.
Fertility preservation for cancer patients	If a partner is affected with cancer, the couple can undergo an ART cycle and have their embryos frozen for their own future use.

Table 4: Indications for embryo cryopreservation

Risk of cryopreservation

While cryopreservation is a boon to ART, it also carries with it certain risks such as loss of cell viability, DNA damage, free radical generation and decrease in fertilizing potential of the gametes and reduced implantation potential of the embryos. These exogenous non-physiological processes can change the genetic and epigenetic integrity of the gametes or embryos. Though there are not many reports, the available literature suggests that there is no difference in the post-natal developmental characteristics of progenies born from cryopreserved gametes or embryos.

The same however cannot be said for gonadal tissue as the fertility restoration is still in its infancy. Another major concern is the transmission of infectious agents through liquid nitrogen. It has been shown that infectious agents such as viruses can cross contaminate other stored samples either due to breakage of straws or leakage of samples from sealed ends of plastic straws.[55] The risk of transmission can be significantly reduced by storing the infected specimens in separate liquid nitrogen containers designated for infectious samples.

Conflict of interest: Authors declare no conflict of interest.

References

1) Triana V. Artificial insemination and semen banks in Italy. In: G. David and W Price.editor. Human Artificial Insemination and semen preservation. Newyork, Plenum; 1980. p51,.

2) Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature,1949;15;164(4172):666.

3) Bunge RG, Keettel WC, Sherman JK. Clinical use of frozen semen. FertilSteril. 1954; 5(6): 520-9.

4) Bunge RG, Sherman JK. Fertilizing capacity of frozen human spermatozoa. Nature.1953; 24;172(4382):767-8.

5) Mazur P. Freezing of living cells: Mechanisms and implications. AmJPhysiol. 1984;247(3 Pt 1):C125-42.

6) Carroll J, Wood MJ, Whittingham DG. Normal fertilization and development of frozen thawed mouse oocytes: Protective action of certain macromolecules. BiolReprod. 1993;48:606-612.

7) Dumoulin JC, Bergers-Janssen JM, Pieters MH, Enginsu ME, Geraedts JP, Evers JL. The protective effects of polymers in the cryopreservation of human and mouse zonae pellucidae and embryos. Fertil Steril.1994;62(4):793-8.

8) Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. BiolReprod.2002;67(6):1671-80.

9) Jelinkova L, Selman HA, Arav A, Strehler E, Reeka N, Sterzik K. Twin pregnancy after vitrification of 2-pronuclei human embryos. Fertil Steril.2002; 77(2):412-4.

10) Selman HA, El-Danasouri I. Pregnancies derived from vitrified human zygotes. Fertil Steril.2002; 77(2):422-3.

11) Yokota Y, Sato S, Yokota M, Ishikawa Y, Makita M, Asada T, et al. Successful pregnancy following blastocyst vitrification. Hum Reprod. 2000; 15(8): 1802-3.

12) Mukaida T, Nakamura S, Tomiyama T, Ishikawa Y, Makita M, Asada T, et al. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop container-less technique. Fertil Steril. 2001;76(3):618-23.

13) Shaw JM, Cox SL, Trounson AO, Jenkin G. Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. Mol Cell Endocrinol. 2000; 161(1-2): 103-10.

14) Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology. 2000b; 53(1): 59-72.

15) Gao DY, Mazur P, Critser JK. Fundamental cryobiology of mammalian spermatozoa. In: Karow A,editor. Critser JK, Reproductive Tissue banking. Academic Press, San Diego, CA.1997; 263-328.

16) Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function.Reprod Fertil Dev.1995; 7(4): 871- 891.

17) Wooley DM, Richardson DW. Ultrastructural injury to human spermatozoa after freezing and thawing. JReprod Fert. 1978; 53(2): 389-94.

18) Henry MA, Noiles EE, Gao D, Mazur P, Crister JK. Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. Fertil Steril. 1993;60(5):911-8.

19) Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology. 2002;57(1):327-44.

20) O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002;17(3):704-9.

21) Duru NK, Morshedi M, Schuffner A, Oehninger S. Cryopreservation-thawing of fractionated spermatozoa and plasma membrane translocation of phosphatidylserine. Fertil Steril. 2001; 75(4): 263-8.

22) Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril. 1979; 31(5): 531-7.

23) Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl.1987; 8(5):338-48.

24) Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil.1987; 81(2):459-69.

25) Van Loon AA, Den Boer PJ, Van der Schans GP, Mackenbach P, Grootegoed JA, Baan RA, Lohman PH. Immunohistochemical detection of DNA damage induction and repair at different cellular stages of spermatogenesis of the hamster after in vitro or in vivo exposure to ionizing radiation. Exp Cell Res. 1991; 193: 303-309.

26) Sanders JE, Buckner CD, Amos D, Levy W, Appelbaum FR, Doney K, Storb R, Sullivan KM, Witherspoon RP, Thomas ED. Ovarian function following marrow transplantation for aplastic anaemia or leukaemia. J Clin Oncol. 1988; 6(5): 813-8.

27) Wallace W, Shalet S, Hendry J, Morris-Jones P, Gattamanemi H. Ovarian failure following abdominal irradiation in childhood; radiosensitivity of the human oocyte. Br J Radiol. 1989; 62: 995-998.

28) The Assisted reproductive technologies regulation bill 2010. ICMR. Ministry of health & Family Welfare. Govt. of India. 2010.

29) Carroll J, Depypere H, Matthews CD. Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. J Reprod Fert.1990;90(2):47- 53.

30) Pickering SJ, Johnson MH. The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. Hum Reprod. 1987; 2(3): 207-216.

31) Aigner S, Van der Elst J, Siebzehnrubl E, Wildt L, Lang N, Van Steirteghem AC. The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. Hum Reprod. 1992; 7: 857-864.

32) Johnson MH, Pickering SJ, George MA. The influence of cooling on the properties of the zona pellucida of the mouse oocyte. Hum Reprod. 1988; 3, 383- 387.

33) Schalkoff ME, Oskowitz SP, Powers RD. Ultrastructural observations of human and mouse oocytes treated with cryopreservatives. Biol Reprod. 1989; 40: 379-393.

34) Glenister PH, Wood MJ, Kirby C, Whittingham DG. Incidence of chromosome anomalies in first-cleavage mouse embryos obtained from frozen-thawede oocytes fertilized in vitro. Gamete Res. 1987;16(3):205-16.

35) Bouquet M, Selva J, Auroux M. The incidence of chromosomal abnormalities in frozen-thawed mouse oocytes after in-vitro fertilization. Hum Reprod. 1992; 7(1): 76-80.

36) Gook DA, Osborn SM, Johnston WI. Parthenogenetic activation of human oocytes following cryopreservation using 1,2-propanediol. Hum Reprod. 1995; 10(5): 654-8.

37) Oktay K, Karlikaya GG, Aydin BA. Ovarian cryopreservation and transplantation: basic aspects. Mol. Cell Endocrinol. 2000; 169(1-2): 105-8.

38) Ben-Yosef D. Yogev L, Hauser R, Yavetz H, Azem F, Yovel I, et al. Testicular sperm retrieval and cryopreservation prior to initiating ovarian stimulation as the first line approach in patients with non-obstructive azoospermia. Hum Reprod. 1999; 14(7):1794-801.

39) Osmanagaoglu K, Vernaeve V, Kolibianakis E, Tournaye H, Camus M, Van Steirteghem A, Devroey P. Cumulative delivery rates after ICSI treatment cycles with freshly retrieved testicular sperm: a 7-year follow-up study. Hum. Reprod. 2003; 18(9): 1836-40.t

39) Osmanagaoglu K, Vernaeve V, Kolibianakis E, Tournaye H, Camus M, Van Steirteghem A, Devroey P. Cumulative delivery rates after ICSI treatment cycles with freshly retrieved testicular sperm: a 7-year follow-up study. Hum. Reprod. 2003; 18(9): 1836-40.

40) Nicopoullos JDM, Gilling-Smith C, Almeida1 PA, Ramsay JWA. The results of 154 ICSI cycles using surgically retrieved sperm from azoospermic men. Hum. Reprod. 2004; 19(3): 579-85.

41) Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WH. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults.Lancet Diabetes Endocrinol. 2015 Jul;3(7):556-67.

42) Ethics committee of ASRM. Fertility preservation and reproduction in patients facing gonadotoxic therapies: an Ethics committee opinion. Fertil Steril. 2018;110(3):380-6.

43) Meistrich ML. Male gonadal toxicity. Pediatric Blood Cancer. 2009;53(2):261-6.

44) Guidice MG, Michele de F, Poels J, Vermeulen M, Wyns C. Update on fertility restoration from prepubertal spermatogonial stem cells: How far are we from clinical practice? Stem cell Res. 2017;21:171-7.

45) Wood CE, Shaw JM, Trounson AO. Cryopreservation of ovarian tissue - Potentail 'reproductive insurance' for women at risk of early ovarian failure. Med J Aust. 1997;166(7):369.

46) Oktay K, Buyuk E. The potential of ovarian tissue transplant to preserve fertility. Expert Opin BiolTher. 2002; 2: 361-70.

47) Aubard Y, Piver P, Teissier MP. Indications de la cryopreservation du tissue ovarien. La Presse Medicale 2000; 29: 960-4.

48) Cohen J,De Vane GV, Elsner CW, Fehilly CB, Kort HI, Massey JB, Turner TG. Cryopreservation of zygotes and early cleaved human embryos. Fertil. Steril. 1988; 49: 283-9.

49) Salumets A, Tuuri T, Makinen S, Vilska S, Husu L, Tainio R et al. Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen-thawed embryo transfer. Hum. Reprod. 2003; 18(9):1890-5.

50) Lassalle B, Testart J, Renard JP. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. Fertil Steril. 1985; 44(5): 645-51.

51) Cohen J, Simons RF, Edwards RG, Fehilly CB, Fishel SB. Pregnancies following the frozen storage of expanding human blastocysts. J Vitro Fert. Embryo Transfer 1985; 49(2): 59-64.

52) Mohr LR, Trounson AO. Cryopreservation of human embryos. Ann. N. Y. Acad Sci. 1985; 442(1): 536-43.

53) Quintans CJ, Donaldson MJ, Bertolino MV, Pasqualini RS. Birth resulting from transfer of blastocysts cryopreserved with propanediol after spontaneous hatching. Reprod Biomed Online 2003; 6: 66-8.

54) Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005; 11(3):300-8.

55) Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, Irwin D, Blair S, Gorman AM, Patterson KG. et al. Hepatitis B transmission from contaminated cryopreservation tank. Lancet 1995; 346(8968): 137-40.