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Clinical Outcomes of Assisted Reproductive Techniques Using Cryopreserved Gametes and Embryos in Human Medicine

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.80627>

Abstract

The methods of cryopreservation play a key role in assisted reproductive technique (ART) treatments, as they increase the efficacy of the treatments by allowing banking of supernumerary embryos for later use. It has been recently proposed that these methods could also increase the safety of ART treatments, by reducing complications such as ovarian hyperstimulation during early pregnancy; thus, the policy of total freeze for later differed transfer of embryos has been proposed. Also of great importance, cryopreservation of oocytes and spermatozoa has permitted gamete storage for long term facilitating practical routines such as the gamete banking for third-party reproductive treatment. In this chapter, the clinical indications and treatment outcomes will be revised and data updated on the safety of using cryopreservation methods in ART treatments.

Keywords: cryopreservation methods, assisted reproductive technique (ART) treatment, pregnancy, embryos, ovarian hyperstimulation

1. Introduction

The development of cryopreservation techniques has made possible the use of frozen and thawed gametes and embryos aiming at reproduction, by means of assisted reproductive techniques (ART). Cryopreservation allows to banking gametes for later use, including also the possibility to be used by other individuals, as in donor treatments. Effective techniques such as in vitro fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI), worldwide

applied, offer a high efficacy by the creation of supernumerary embryos. As recognised downsides of IVF/ICSI treatments include the high prevalence of perinatal complications due to multiple births, the recommended practice of transferring fewer embryos in the fresh IVF treatment cycle, with the goal of performing single embryo transfer and the cryopreservation of remaining embryos for their later use in frozen-thawed cycles, one at a time, is currently the trend [1]. The cumulative chance to achieve pregnancy and live-birth through IVF/ICSI treatments is thus enhanced by the later use of thawed embryos in separate treatments.

The methods for cryopreservation of embryos and gametes have demonstrated effective and safe, and have developed towards the achievement of a clinically established level. These methods are also currently being offered to patients suffering of cancer, due to the risk of infertility associated with certain cancer treatments, or to individuals with conditions that have an inherent risk of premature gonadal insufficiency and infertility, aiming at fertility preservation [2].

Although embryo cryopreservation has historically been regarded as the first-choice technique for fertility preservation, social, ethical and legal reasons usually restrict its use to couples who have entered into a committed long-term relationship. However, women without a partner may attempt this possibility using a sperm donor. Furthermore, this issue has also been shown to translate to fertility preservation undertaken electively, with one study finding that >80% of patients undergoing oocyte preservation by choice were single at the time, and that lack of a partner was by far the most common reason for not pursuing child-bearing earlier [3].

As such, the cryopreservation of gametes affords individuals an increased level of reproductive autonomy, and ensures that fewer patients are faced with the extraordinarily difficult decision of later reproducing with a partner who may no longer be ideal, or not reproducing at all. Here, the most common indications for gamete cryopreservation in both males and females and embryo cryopreservation will be discussed, along with their clinical outcomes, necessary considerations and future perspectives.

2. Current status of fertility preservation by cryopreservation of gametes

Although semen cryopreservation has remained an established technique for many years, the cryopreservation of mature oocytes was considered experimental by the American Society for Reproductive Medicine (ASRM) until 2013 [4]. As such, this relatively recent development has paved the way for an explosion of social fertility preservation ('social freezing'), found by a HFEA report to have increased more than two-fold between 2013 and 2016. In fact, a 10% increase in the number of egg freezing cycles was reported from 2015 to 2016 [5]. In a field which was once dominated by fertility preservation following medical diagnosis, this represents a dramatic paradigm shift, which must be regulated to ensure transparency for, and protection of, the prospective patient. This differs from traditional approaches to non-elective fertility preservation, where patients who may be younger or have no desire to delay child-bearing are faced with a high likelihood of complete infertility following resolution of their disease. Therefore, such conditions encompass those that directly cause premature ovarian

insufficiency (POI) such as bilateral benign ovarian tumours, severe recurrent endometriosis or genetic disorders (e.g., Turner's syndrome), and conditions that indirectly result in POI such as malignant or non-malignant diseases that require the administration of gonadotoxic chemo- or radiotherapy [6].

Whilst there is a tendency to focus primarily upon female infertility due to the intrinsically finite nature of female reproductive biology, it must be remembered that males are also distinctly susceptible to gonadotoxic agents, with one study reporting that up to 60% of male cancer survivors experience fertility impairment [7]. In fact, malignant diseases are amongst the most significant indirect contributors to infertility worldwide, with some of the most commonly-used classes of chemotherapeutics, alkylating agents, having been shown to induce POI in 42% of women treated [8]. The situation is further complicated by the widespread use of novel targeted therapies whose impact upon fertility is largely unknown [9]. These advances in cancer treatment efficacy (coupled with societal pressures to delay childbearing) have led to an increasing proportion of cancer survivors who wish to further add to their families, resulting in increased public awareness of treatment-induced subfertility, increased demand for fertility-preserving procedures, and the emergence of a brand-new field: oncofertility [10, 11]. This new discipline is badly-needed, providing patients with essential information that will impact upon their treatment decisions and future family planning, and aiming to disrupt the traditional lack of emphasis placed on iatrogenic infertility in the oncological sphere [11–13].

Another newly-emerging paradigm in gamete cryopreservation is its implementation as a timesaving method in fertility treatment. Age is the most significant determinant of IVF cycle outcome, meaning that older females who present for treatment may be considered for multiple consecutive rounds of ovarian stimulation and egg collection, thereby facilitating the freezing of large numbers of eggs which can later be fertilised and transferred [14]. This is a significant advantage for couples who may want multiple children, or who find the storage of a large number of embryos ethically questionable. It is open to debate whether this application should be considered medical or social, but as technology advances, it is important we consider such applications that lie within the 'grey areas' of medicine.

2.1. Particular considerations regarding the cryopreservation of spermatozoa

Sperm cryopreservation is the only established fertility preservation method in post-pubertal males, and has been in clinical use for over 50 years [15]. Its early adoption to the clinical realm is attributed to the accidental discovery of the cryoprotective properties of glycerol on sperm cells, their abundance for experimental uses and their small size. This latter property is an extremely important one, reducing the likelihood of damaging intracellular ice crystal formation during the freezing process. Whilst cryopreservation by slow freezing protocol was the first method used successfully, it causes extensive chemical and physical damage to sperm cell membranes, with only 60% of sperm regaining motility post-thaw [16]. Comparative studies have demonstrated that non-standard methods of rapid freezing (vitrification) using liquid nitrogen give better post-thaw motility rates and alter protein expression profiles less, as well as being more time- and cost-efficient [17, 18]. Whilst both methods result in

a significant reduction in viability, the (generally) high number of spermatozoa per sample means that lower survival rates are acceptable. As such, either cryopreservation method may be used effectively. This relatively low bar for post-thaw viability contrast hugely with oocyte cryopreservation, where the numbers of gametes collected tends to be small, and therefore more stringent protocols and attrition rates are required.

Sperm samples for cryopreservation are usually obtained by masturbation, but if in the cases of azoospermia, males who are unable to provide a sample (e.g., for psychosocial or physical reasons) or those who have previously undergone a vasectomy, surgical techniques may be employed. These include epididymis aspiration, testicular needle biopsy (TESE) or needle aspiration (TESA), with TESE having impressive success rates of 85%, even following chemotherapy for testicular cancer [19]. It is important to note that although these methods of sperm retrieval are effective, all require that the patient is able to produce spermatozoa, even at dramatically decreased levels. Options are extremely limited for patients whose Sertoli cells are non-functional, or pre-pubertal males, with the cryopreservation and autotransplantation of spermatogonial stem cells (SSCs) still classified as experimental, but showing promise in animal models [20]. In vitro maturation of SSCs, or SSC derivation from induced pluripotent stem cells (iPSCs) are also avenues under investigation [21].

Whilst both the American Society of Clinical Oncology (and almost all other) guidelines recommend that fertility preservation be offered to pubertal males before commencement of gonadotoxic treatment, only 25% of eligible males in the relevant cohorts bank sperm. These statistics are surprisingly low, especially when one considers the generally non-invasive nature of semen sample collection, and the wealth of prospective studies supporting that viewpoint that the overwhelming majority of men diagnosed with cancer wish to have children later in life [22]. One such study reported that 43% of patients surveyed ranked reproducing as a 'top 3' life goal [23]. It is therefore apparent that a disconnect exists in male fertility preservation that is not present to the same degree in the female equivalent. This may be due to routinely poor counselling by clinicians, but it is also possible that the priorities of young male patients may not adequately reflect their later life goals, or that male stoicism might affect the decisions made. Equally, the perceived high cost of cryopreservation and storage might have a role to play, even though robust cost-benefit analyses have shown sperm cryopreservation to be more cost-effective than post-therapeutic fertility management [19]. It has been evidenced that long-time storage does not seem to affect the fertilisation potential of sperm, as recently reported after 40 years of storage [24].

2.2. Particular considerations regarding the cryopreservation of oocytes

In contrast to spermatozoa, mature (MII) oocytes are large, fragile cells that are much more susceptible to water retention and ice crystal-mediated damage. Furthermore, addition of cryoprotectants may result in osmotic stress, with the cumulative effect of these stressors manifesting as thickening of the zona pellucida, premature cortical granule exocytosis and meiotic spindle disruption [25, 26]. Although this disruption of the meiotic spindle appears to be transient in almost all cases, there is robust evidence to show that cryopreservation negatively impacts oocyte gene expression and proteomics, with some cryoprotectants even shown to alter maternally-derived proteins which support early oocyte development [27–29].

The net result of this is a 'stressed' oocyte which is difficult for spermatozoa to penetrate and fertilise. As such, the clinical applications of oocyte cryopreservation were limited until the inception of the ICSI technique in 1992 [30], with the first pregnancy derived from frozen oocytes following in 1997 [31].

Another quantum leap forward in the efficacy of oocyte cryopreservation came with refinement of freezing protocols. Similar to the paradigm change seen in spermatozoa cryopreservation, vitrification (fast freezing) techniques were pioneered, first producing a live birth in 1999, and then being further improved by Japanese groups in 2003 [32, 33]. In contrast to the small increase in efficacy seen with the introduction of vitrification in spermatozoa cryopreservation, however, vitrification of oocytes seems to greatly increase post-thaw oocyte survival and fertilisation rates, with a 2014 Cochrane review finding a relative increase in oocyte survival of 29%, and a 19% increase in fertilisation [34]. An additional meta-analysis of three RCTs in 2016 reported a 16.1% increase in survival (RR = 1.23, 95% CI: 1.02–1.49; P = 0.031) [35]. The efficacy of the vitrification technique was further confirmed when a large prospective study of Spanish egg-donation programmes could not detect any statistically significant difference between using fresh donor eggs, when compared to vitrified frozen eggs [36]. It is important to note, however, that both of these techniques are inherently operator-dependent; with vitrification especially variable due to the need to complete the process within seconds [37]. This is an important caveat, and highlights the importance of training and upskilling, especially when considering the variable experience that operators may have within the same fertility clinic. It must also be clarified that the survival rates of oocytes (and the number collected) are likely dependent on the age and disease status of the donor, meaning that the extremely high survival rates of thawed oocytes reported by some studies on donor eggs (in excess of 96%), may not be truly representative for a significant proportion of patients who undergo fertility-preserving treatment [38].

It is clear, therefore, that the path to the clinic for oocyte cryopreservation has not been a straightforward one, with the early, highly-ineffective methods of oocyte cryopreservation making it an unrealistic and imprudent option for females in urgent need of fertility preservation, such as oncology patients. Cancer in reproductive age is twice as common in females as in males, and more than half of those diagnosed are expected to undergo treatment that compromises their fertility [39]. One large retrospective study highlighted this, indicating that whilst the incidence of treatment-related acute ovarian failure (AOF) was approximately 10%, these figures greatly misrepresent the total age-specific impact on fertility, with 40% of those not reporting AOF encountering infertility by the age of 35 [40]. Furthermore, the probability of early menopause was 'at least' 25% by age 30 [40]. It is likely, therefore, that effects on fertility may often relate to a reduction in the overall number of primordial follicles, and may therefore remain undetected until later in life. In a society where increasing numbers of women are choosing to delay childbearing, this may mean that women who are presumed to have normal reproductive activity following resumption of menstruation may not try to conceive as early as they are able to, and then later encounter difficulty.

It is important to consider that patients undergoing fertility-compromising cancer treatments may only have sufficient time for one round of ovarian stimulation and egg collection before their treatment must begin. This process of controlled ovarian stimulation (COS) followed by

egg collection generally takes approximately 2 weeks to complete, with patients able to start chemotherapy within 48 h of completion. Whilst concerns had initially been raised about the administration of such high doses of exogenous gonadotrophins to patients with hormone-sensitive cancers (e.g. breast, ovarian), effective and safe stimulation protocols using aromatase inhibitors have been developed and shown to result in no increased risk of recurrence in breast cancer, after a mean 5-year follow-up period [41]. In addition, the use of GnRH antagonist regimens (in place of the usual GnRH agonist regimens) allow ovarian stimulation to be started at any point in the menstrual cycle ('random-start protocols'), thereby minimising treatment delays. These GnRH antagonist regimens have been shown to result in the collection of similar numbers of mature oocytes and produce similar fertilisation rates [42]. Moreover, they have been shown to result in a lower risk of ovarian hyperstimulation syndrome (OHSS) than conventional protocols [43].

As such, refinements in cryopreservation techniques and stimulation protocols represent incredibly important steps for cancer (and elective) patients, increasing both the safety of oocyte collection and the likelihood of a live birth following completion of treatment.

2.3. Clinical outcomes of using cryopreserved gametes

As outlined above, the cryopreservation of gametes is a technically difficult and expensive process. As such, it is essential that the true success rates of these procedures be analysed using clinical endpoints, in order to prevent delays to treatment, unnecessary harm to patients and to disrupt the growing belief amongst the general proportion that egg freezing constitutes an infallible 'insurance policy' against age-related fertility decline.

In order to assess the success of cryopreservation we must first examine the parameters by which success is gauged. The most realistic way to evaluate the efficacy of cryopreservation techniques (and indeed individual clinics) is through the comparison of live births achieved per oocyte thawed. Although this may seem obvious, there is a growing propensity for some clinics (especially those who derive a significant proportion of their income from social egg freezing) to display these statistics in a manner that makes them appear more impressive. For example, some success rates might be represented using clinical pregnancy rates per thaw cycle; with some studies reporting this to be as high as 78% [44]. This figure is not an accurate representation of the reality faced by most patients, with the largest reported study of 3610 vitrified oocytes producing an oocyte survival rate of 90%, translating to a clinical pregnancy rate of 48% and an 'oocyte-to-baby' rate of just 6.5% [45]. If this same study were to be presented alternatively, it could be quoted as a delivery rate of 78.8% per oocyte donation cycle. As such, it is clear that there must be further efforts to homogenise how 'success' is calculated, and increased scrutiny of how these results are presented to potential patients. It is essential, also, to note that this data (and indeed almost all data on oocyte cryopreservation) has been generated from oocyte donation programmes. This is significant because oocyte donors tend to be carefully-selected, young individuals, whose eggs are likely to be of greater quality than the average patient wishing to engage in autologous fertility preservation. In fact, this viewpoint is supported by findings that only 32% of patients freezing their eggs were below the age of 35, and recent data showing reduced yield of oocytes collected in oncology patients versus matched controls [5, 38, 44]. As such, it is likely that the true likelihood of a successful

live birth for patients in these groups is significantly lower than the figures generated by current data. It is essential, therefore, that the increasing availability of data from non-donation sources be interpreted and used to validate the statistics that are currently quoted.

The largest study using data collected from outside of egg donation programmes was carried out by Cobo et al., who examined the reproductive success of 1468 women undergoing elective oocyte cryopreservation for non-oncologic reasons [46]. Their data clearly demonstrates the impact of age at freezing upon potential success, with those who froze at or before the age of 35 having a 53.9% likelihood of a live birth per ET, whilst those freezing at or above the age of 36 had a 22.9% chance. This viewpoint was echoed by a recent HFEA report, who described patient age at freezing as 'the most important factor', whilst age at thaw was not determined to have any statistically significant impact [5]. The same study also demonstrated the importance of the number of oocytes obtained to vitrify in increasing chances of a live birth, with an increase from 5 to 8 oocytes producing the most significant increase in LBR (8.4% per oocyte if <35). Whilst an average 'oocyte-to-baby' ratio is omitted, it is estimated to be significantly lower than the 6.5% achieved in donor programmes. Whilst this is an interesting figure, it is likely that it does not provide as clear a picture of the factors that impact oocyte viability as that provided by age-bracket stratification.

Consequently, we can conclude that the number of viable oocytes available for fertilisation is a clear determinant of the likelihood of successful pregnancy. The technique used to freeze and thaw the oocytes retrieved is thus of the utmost importance, with a multitude of studies confirming the advantages provided by vitrification protocols, both in terms of post-thaw oocyte survival and reported pregnancy rate. In fact, multiple studies reported the clinical pregnancy rate (CPR) to more than double when compared to slow-freezing protocols [47, 48]. In addition, there is increasing scrutiny on the impact that the rate of warming can have on post-thaw oocyte survival and characteristics. In fact, Mazur and Seki reported oocyte survival >80% when ultra-rapid warming was carried out, even when using traditional slow-freeze protocols. Further expanding on this, they demonstrated that such methods could be used to reduce the concentrations of cytotoxic chemoprotectant required for the vitrification process [49]. Interestingly, a recent meta-analysis of five studies concluded that there was no significant difference between the fertilisation rates, embryo cleavage or pregnancy rates achieved when using fresh versus vitrified oocytes [50]. This viewpoint is supported by recent data supplied by the HFEA, who concluded that the birth rate per embryo transfer (PET) was rising to over 19%, and within 2% of the overall IVF birth rate PET [5]. In addition, multiple studies have demonstrated that the length of storage has no effect on pregnancy rates or outcomes [45, 51]. As such, it is reasonable to conclude that cryopreservation techniques have advanced to such a stage that significant future improvements in success rates will likely relate to methods of increasing the yield of oocytes collected per stimulation cycle, or in the methods used to select the embryos to be transferred.

The above discussion is necessarily focused on female gametes, as spermatozoa quality has traditionally been seen to be of less importance owing to the large number usually obtained per collection and their high survival rate. It is also worth noting that studies have found no correlation between sperm quality and disease stage in oncology patients [52]. In addition, the advent of ICSI has meant that even 'poor quality' sperm samples with low motility scores

can be used to produce a viable embryo. That said, it is almost certain that there exist intrinsic variations in spermatozoa quality that currently evade detection, driving increased research into how we select the sperm we use for fertilisation, both in the context of conventional IVF, and in fertility preservation. The artificial techniques discussed have abrogated the physiological selection methods inherent to the natural reproductive process, paving the way for a growing need for the 'unnatural selection' of favourable gametes via novel biomarkers or growth characteristics. Ongoing avenues of such research include the assessment of spermatozoal DNA fragmentation rates (although evidence is not yet conclusive), and promising future avenues such as the stratification of sperm quality via spectrophotometric analytical techniques such as Raman spectroscopy [53]. In fact, the latter method would allow andrologists to select spermatozoa on the basis of both their homeostatic and epigenetic context [54].

It follows that an essential aspect of any discussion on the clinical outcomes of gamete cryopreservation must be that of perinatal outcomes. It is often easy to rely on pregnancy rate as the sole benchmark of a successful preservation cycle, but serious consideration must also be given to whether the progeny created are morphologically, genetically and developmentally 'normal'. Reassuringly, a number of analyses, one of 165 pregnancies and another of 936 infants, have found a comparable incidence of congenital abnormalities in infants born following oocyte vitrification, conventional IVF and natural pregnancy [55, 56]. There is also a growing body of evidence, however, that IVF may trigger epigenetic disruption in the developing embryo, potentially causing the slightly lower birth weights observed amongst children born as a result of these techniques [57]. That said, it is also possible that these differences are related solely to the increased ages of the patients within the IVF cohort. A long-established relationship exists between increased parental age and genetic dysfunction, with increased maternal age being linked to abnormal meiotic spindle function, and therefore the induction of gross chromosomal abnormalities such as Trisomy 21 (Down's syndrome) [58]. Similarly, it has been shown that the higher prevalence of single point mutations seen in children born to fathers of more advanced age is attributable to the higher number of mitotic replications that these germ cells have experienced [59]. It is thought that this is a direct cause of the increased rates of neurodevelopmental disorders, leukaemias and stillbirths seen in this paternal cohort [60]. As such, although age has a strong positive correlation with adverse perinatal outcomes, no cryopreservation-specific (or indeed fertility treatment-specific) causal relationship has yet been reliably established.

In fact, the most common perinatal outcomes that are directly attributable to IVF are due to multiple pregnancies. These usually occur as a consequence of the transfer of more than one embryo, and may result complications such as premature birth, intrauterine death and conversion to caesarean section [61]. Whilst this, and complications associated with advanced maternal age, certainly remain considerations in the fertility preservation sphere, the patients concerned tend to have fewer options and less time to achieve a successful pregnancy, making the delivery of multiple children more serendipitous than it otherwise might be. Indeed, as the average age of childbearing increases (due, in part, to ART), it is arguable that discussion of such 'difficult pregnancies' will be of less future importance, as prospective patients will almost always opt to try to conceive in the face of an increased risk of poor perinatal outcome, instead of not attempting to conceive at all.

In conclusion, although the oocyte conversion rates discussed above might seem extremely poor at the outset, it must again be stressed that modern assisted reproduction technologies circumvent the physiological selection mechanisms that serve to ensure only the most viable gametes survive. Success rates using cryopreserved gametes are almost comparable to those achieved using fresh gametes, and therefore it is reasonable to expect the efficacy of both techniques to advance in parallel as our knowledge and gamete selection methods improve. The **Table 1** presents several methods currently used to improving gamete selection for cryopreservation.

2.4. Societal and ethical aspects of cryopreserving gametes

Although the technical aspects of gamete cryopreservation have been discussed at length above, one must also consider the societal and ethical impact of such procedures. Gametes are incredibly prized cells; holding the genetic information is required to produce related offspring for those at high risk of fertility disruption. Therefore, the conditions under which they are stored, the individuals permitted to handle them, access related information whilst they

| Technique | Description | Evidence |
|--|--|---|
| Pre-freeze swim-up preparation (spermatozoa) | Traditionally, sperm selection via preparative techniques was undertaken post-thaw. There is increasing evidence, however, to show that such swim-up techniques should be performed before cryopreservation to produce the highest percentage of viable spermatozoa. It is theorised that cytokine release from immune cells that are inadvertently included in cryopreserved samples may damage spermatozoa quality, and that this could be avoided using these pre-freeze techniques. This viewpoint has been supported by data from recent trials. | Petyim et al. [111] |
| Rate of cooling | As evidenced by the aforementioned increases in gamete quality using vitrification techniques, the rate of cooling during cryopreservation is extremely important. As such, efforts have been made to dramatically decrease the volume of the solution in which oocytes are vitrified (now 0.1–2 µL). To facilitate this, specialised carriers have been developed, including both open and closed systems. Comparative analysis of these two categories of systems has demonstrated similar oocyte survival rates, but significantly increased cytoplasmic vesicle presence (and theorised reduction in quality) in oocytes frozen using the closed system. | Bonetti et al. [112] |
| Low-CPA protocols | Protocols that employ low concentrations of cryoprotectants have the potential to combine the positive aspects of vitrification and slow-freezing, without their respective associated disadvantages. Although such protocols have been impractically complex and time-consuming, recent advances in quartz micro-capillary techniques are showing promise. | Choi et al. [113] |
| Single-gamete analysis | Although not yet adequately optimised for clinical use, analysis of individual gametes has the potential to revolutionise how ART is carried out. The increasing need for artificial selection has meant that there is now increasing scrutiny on spectrophotometric and other non-invasive analytical techniques, some of which have been shown to provide adequate comparative analysis for oocyte quality and sperm DNA fragmentation rate. Whether this comparative analysis will be of clinical use, however, remains to be seen. | Davidson et al. [114] Sanchez et al. [115] |

| Technique | Description | Evidence |
|---|---|--|
| In-vitro maturation (IVM) of immature oocytes | IVM aims to increase the yield of oocytes available for cryopreservation through the obtaintion of additional M2 oocytes from oocytes that would otherwise be discarded. Although data shows that approximately 35% of IVM oocytes can produce cleavage-stage embryos when fertilised, and this method has been suggested to increase the efficacy of treatment cycles aimed at fertility preservation, there is currently insufficient data to support the systematic use of IVM techniques or the freezing of immature oocytes. | Oktay et al. [116] Phoon et al. [117] |
| Selection via DNA fragmentation rate | It is clear that a both vitrification and slow-freeze protocols produce DNA lesions, either via full or partial fragmentation. Although modern analytical techniques can quantify this fragmentation (and resulting apoptotic induction), they most commonly result in destruction of the gamete in question. As such, although they may provide valuable information on the quality of a particular sample, they do not provide a solution for the accurate selection of gametes which may prove more viable that their morphologically-normal counterparts. | Valcarce et al. [118] |
| CPA equilibration temperatures | Changing the equilibration temperature with CPA and increasing the sucrose concentration added have both proven to be effective strategies to improve oocyte survival and fertilisation rates, respectively | Borini et al. [119] |

Table 1. Methods of improving gamete selection when employing cryopreservation techniques.

are stored, and the length of time which they can be stored for are of the utmost importance. Although legislative circumstances may vary from country to country, the HFEA permits storage of gametes or embryos for an initial maximum period of 10 years, with this being extended by 10 years at a time on a case-by-case basis up to a maximum of 55 years [61]. These limits are important to protect the wellbeing of prospective children, and to prevent the misuse of genetic material. Furthermore, as technological advances in genetics allow increasingly accurate prediction of phenotype and disease likelihood, it is likely that the genetic material contained within gametes will need progressively more stringent protection. An example of such measures includes the recently-enacted General Data Protection Regulation (GDPR), which legislates for the prevention of the misuse of such genetic data [62].

The societal effects of the growing popularity of cryopreservation must also be considered. More women than ever before are experiencing the ironic dichotomy of spending the vast majority of their reproductive years trying no ensure that they do not fall pregnant, but then finding themselves unable to conceive when they try to. As such, the landscape of this exploding field is increasingly commercial, providing increased funds to facilitate advances in treatment efficacy at the cost of advertising cryopreservative services as an insurance policy against age-related fertility decline. There is also a worrying increase in the number of companies offering ‘social freezing’ as part of their employee benefit packages. This is a trend that propagates the misinformed idea that social cryopreservation guarantees a later pregnancy, and serves to perpetuate the societal pressure placed on women to delay childbearing [63]. The cost of such procedures (if not covered by insurance or a third party) is also a valid consideration, with various cost benefit analyses finding contrasting conclusions on whether it is more, or less cost-effective to cryopreserve in one’s mid-twenties and return to them at age 40,

or just to attempt conventional IVF at age 40 [14, 64]. Whilst this is an important avenue of discussion, the superior success rates provided by the cryopreservation route are likely to provide a superior chance of obtaining a live birth.

While the risks associated with childbearing at an increased age may have the immediate downstream effects of reducing the incidence of certain genetic aberrations, it is also important to consider knock-on effects which may not be immediately obvious. It is possible that widespread societal gamete cryopreservation could unearth harmful novel ART-mediated epigenetic alterations, or further promote the delay of childbearing age. Such effects would doubtless affect the composition of our society, and the manner in which it functions. Therefore, the future direction and regulations governing this area must be scrutinised to determine what should, and should not be permitted. This is a more complex ethical discussion that falls outside of the scope of this chapter, but should nonetheless be kept in mind.

3. Current status of embryo cryopreservation

Since the early days of in vitro fertilisation (IVF) 40 years ago, there have been remarkable advances in clinical and laboratory areas that have opened the door to different variants of standard IVF procedure [65, 66]. Improvements of ovarian stimulation protocols enable the collection of several mature oocytes, which associated with the improvement of the IVF techniques and optimization of embryo culture result in the obtention of a large number of embryos. Therefore, embryo cryopreservation was a necessary evolutionary step for IVF-treatments with the first pregnancy after transfer of a frozen-thawed embryo being reported in 1984 [67]. Since then, embryo cryopreservation has become a widely used technic in assisted reproductive technology (ART), allowing the preservation of the remaining embryos following a fresh transfer for future pregnancies and as a modern tool to reduce multiple births by encouraging patients to transfer a single embryo [1, 68]. Additional indications for embryo cryopreservation are the embryo banking for preimplantation genetic screening, elective deferred embryo transfer, when the patient is at risk of a hyperstimulation and for fertility preservation [66, 69]. Thus, embryo cryopreservation greatly increased the safety and efficacy of IVF treatments and enable the later use of all the embryos obtained from a single oocyte pick-up.

Over the years, cryopreservation methods, protocols and stage at time of cryopreservation have changed, improving embryo cryopreservation techniques. Consequently, the number of frozen-thawed cycles increased worldwide [66, 70] with similar or even higher pregnancy rates compared with the transfer of fresh embryo [65, 70]. In Europe, the last report generated from registers by the European Society of Human Reproduction and Embryology (ESHRE) stated that 154,712 frozen-thawed cycles were performed in 2013, increasing the overall life birth rate by 6% [71].

Herein we will resume the evolution of the embryo cryopreservation methods, stage at which cryopreservation is performed and give an overview of the perinatal outcomes of frozen-thawed embryo transfers.

3.1. Methods of cryopreservation applied to embryos

Since the first reports of pregnancy and delivery after transfer of frozen-thawed embryos in the earlies 1980s [67, 72] various protocols of embryo cryopreservation were introduced. They mostly differ from each other in the type and concentration of cryoprotectants, equilibration timing, cooling rates and freezing devices [35]. Regardless of the cryopreservation method used, the goal is to suspend embryos in time by cooling embryos from ambient temperature to -196°C [73]. Nowadays slow freezing and vitrification are the two principal approaches for embryo cryopreservation, although vitrification has become favored over the last decade [35, 74].

In slow-freezing protocol the temperature is decreased sufficiently slowly to allow the adequate cellular dehydration but also minimising the formation of intracellular ice. This is only possible through the use of a programmable freezing machine. With this method, the samples are first exposed to a quick cooling rate of $2^{\circ}\text{C}/\text{minute}$ until they reach -7°C . Then extracellular ice crystal formation is induced manually (seeding) by touching the vial or straw with pre-cooled forceps as far away from the embryos as possible. As consequence, more water leaves the embryo allowing cryoprotectants to enter. After the seeding, the temperature decreases slowly ($0.3\text{--}1^{\circ}\text{C}/\text{minute}$) untill it reaches temperatures approximately -40°C and then rapidly to -150°C with a cooling rate of approximately $50^{\circ}\text{C}/\text{minute}$. The embryos are then stored in liquid nitrogen until use [35, 74].

Although the first pregnancies and birth were obtain with an embryo cryopreserved with dimethylsulphoxide (DMSO) as cryoprotectant [67, 72] births using other cryoprotectants, such as propanediol (PrOH) were soon reported [75, 76]. Since then, this has become the cryoprotectant more widely used in combination with sucrose for embryo cryopreservation by slow-freezing [35] (**Table 2**). The disadvantage of the slow-freezing method is the formation of ice crystals, increasing the risk of cell damage during thawing. Therefore, despite this method has being used for over 30 years in IVF laboratories and considered safe, since the concentrations of the cryoprotectants used to avoid ice crystal formation are low, another technique was developed to improve embryo cryopreservation—the vitrification.

| | Slow freezing | Vitrification |
|------------------------|---|--|
| Cleavage stage embryos | 1.5 M PROH plus 0.1 M sucrose are included in the most commonly used protocols [74] | EG-based method was early proposed for vitrification of cleavage stage embryos [122] DMSO-based method initially reported the lowest survival rate [123] EG/DMSO/sucrose in open or close systems are the most commonly used cryoprotectants |
| Blastocysts | Glycerol and sucrose as cryoprotectants are included in the most commonly used protocols [74] | EG and DMSO were the cryoprotectants used in the first pregnancy reported after blastocyst vitrification [124] EG/DMSO/sucrose in open or close system are also used [97, 125] |

Abbreviations: EG: ethylene glycol; DMSO: dimethylsulphoxide.

Table 2. Commonly used protocols for cryopreservation of cleavage stage human embryos and blastocysts.

With vitrification, the ice formation is almost eliminated since the cells and the extracellular milieu are solidified into a glass-like state [77]. This method has an extremely high cooling rate in the range of 2500–30,000°C/minute till –196°C by immediate exposure to liquid nitrogen [74, 77]. Despite the high concentrations of cryoprotectants that this method requires, its potential toxicity is reduced by the short time of exposure and the small volume of cryoprotectants used [35]. As in the slow-freezing method different cryoprotectants were tested, leading to the current preferred combination of DMSO (15%), ethylene glycol (EG-15%) and sucrose (0.5 M) in a minimum volume ($\leq 1 \mu\text{l}$) [78] (**Table 2**). The biggest difference between vitrification protocols relates to the cooling and storage methods employed, with open system, involving direct embryo contact with the liquid nitrogen, or closed system involving specific devices to avoid direct contact with the liquid nitrogen [35].

With vitrification a laboratory can expect to obtain an increased embryo cryosurvival rate comparing to the slow-freezing method (**Table 3**), as well as a beneficial effect in the clinical pregnancy rate and live-birth rate per embryo transfer [35, 74, 79, 80]. Additionally, vitrification method does not require any specific equipment and is less time consuming compared to slow-freezing. Consequently, many laboratories worldwide have completely replaced slow-freezing with vitrification [35].

3.2. Cryopreservation at cleavage stage vs. blastocyst

The first pregnancy was obtained with an embryo cryopreserved at eight cells stage [67]. Since then the procedure has changed several times, with the current practice being to preserve either at the cleavage stage Day 2 or 3 of culture or at the stage of blastocyst at day 5 or 6 of culture, despite no clear evidence of which strategy is more beneficial for frozen-thawed embryo transfer [81, 82]. Since only few randomised controlled trials (RCTs) have been conducted to determine what stage of development optimises cumulative birth rate for the retrieval cycle, most of the available data about the timing of embryo cryopreservation is derived from outcomes of fresh cycles [73].

Despite the method of cryopreservation and embryo's stage, the embryo selection for cryopreservation is based on their morphology and pre-freezing morphology is directly related with cryopreservation success and efficiency [83].

| | Cleavage stage | | Blastocyst stage | |
|---|--|----------------------|--|-------|
| | Day 2 | Day 3 | Day 5 | Day 6 |
| Morphology before cryopreservation [74, 81, 97] | ≥ 4 blastomeres <25% fragmentation No multinucleate blastomeres | ≥ 6 blastomeres | Blastocysts are scored according to expansion, inner mass and trophectoderm using Gardner scoring system – 3BB or better | |
| Morphology after thawing [74, 120] | $\geq 50\%$ intact blastomeres Higher number of blastomeres after 24 h of culture | | Scoring according to Gardner, as before cryopreservation | |
| Expected survival [74, 121] | 61.4–87.5% with slow freezing 64–94% with vitrification | | 76.3–88% with slow freezing 84–100% with vitrification | |

Table 3. Morphological aspects of embryos before/after cryopreservation and expected cryosurvival.

For cleavage stage embryos, it is recommended that the embryos selected for cryopreservation should have 4 cells at day 2 and 8 cells at day 3, less than 10% of fragmentation, stage specific cell size and no multinucleate blastomeres [84] (**Table 3**). After thawing, embryos with 100% intact blastomeres will have a higher implantation. However, in embryos with 50% or more cells intact post-thaw and with mitotic resumption, the number the cells at the transfer may be more predictive of the embryo's ability to implant than the percentage of cells surviving at the time of thawing [85–87]. These parameters remain the most clinically important criteria to evaluate the implantation rate potential till today [83].

Advances in culture systems have made possible to prolong embryo culture until the embryo reaches the blastocyst stage. Thus, over the last decade blastocyst transfer at day 5/6 of culture has greatly increased and is seen for some as a “natural selection” of the most viable embryo, similar to the process during spontaneous conception [88, 89]. However, the clinical efficacy of blastocyst transfer over cleavage stage transfer is debatable. In fact, in 2016 a Cochrane meta-analysis reported an increase of clinical pregnancy and live birth after blastocyst transfer [82] but 1 year later another meta-analysis did not find any statistical difference in outcomes when comparing the transfer of embryos at the cleavage stage or blastocyst [90], the same results were previously described for cryopreserved embryos [81].

As with cleavage stage embryos, assessment of blastocyst stage cryopreservation outcomes requires attention to variety of factors before cryopreservation and after thawing in addition to methodology. Outcomes have been shown to be dependent on pre-freeze quality of the blastocyst and time required to reach the blastocyst stage [91, 92].

The most commonly used blastocyst grading systems assigns scores to three morphologic aspects of the embryo: quality of inner cell mass—grades A, B, C; quality of the trophectoderm—grades A, B, C and degree of expansion (**Table 3**). Blastocyst score at the time of cryopreservation was associated with survival and implantation rates [93–95]. Other factors that may contribute to outcomes are the day the cryopreserved embryo reached the blastocyst stage [5, 6], whether the blastocoel was collapsed or not prior to cryopreservation, and evidence of blastocoel re-expansion prior to transfer [96, 97].

3.3. Clinical and perinatal outcomes of pregnancies achieved using frozen-thawed embryo transfers

Frozen-thawed cycles increased during the last decade and a concern about the perinatal outcome also have risen [66]. Although embryo cryopreservation is a well established procedure, long-term studies are still sparse [35]. Data are reassuring suggesting that pregnancies obtained from frozen embryos are not associated with an increased perinatal risk compared to fresh transfers [98–104]. Several reviews have indicated a slightly better result when frozen-thawed embryos were used compared to the fresh transfer, with reduced risks of preterm birth, small for gestational age babies, low birth weight babies and pre-eclampsia,

which could be justified by the endocrine milieu of the stimulation when the transfer is made fresh [70, 99, 102, 104].

Furthermore, a systematic review, published recently, confirmed that singleton babies born after the transfer of frozen-thawed embryos have higher weight at birth when compared to babies born after the transfer of fresh embryos, as well as a higher risk of hypertensive disorders during pregnancy [70].

3.4. Future perspective: the freeze-all strategy

To further improve IVF outcomes, it has been suggested to freeze all the embryos obtained in a stimulation cycle and then plan a deferred transfer during a natural cycle or with hormone replacement with exogenous estradiol (E2) and progesterone (P) for endometrial priming [105, 106]. With this strategy, the frozen-thawed embryos are transferred into a more “physiological milieu” which seems to improve implantation and outcomes compared to fresh transfer [106–108]. However, almost all the data was obtained in patients with high ovarian response patients and thus it was suggested that the freeze all strategy should be performed on patients with a risk of ovarian hyperstimulation syndrome (OHSS), since it was not clear if normal and poor responders will be the same benefits from freezing all the embryos [109].

A recent retrospective study using the general population has reported that 50.74% of patients using the freeze-all strategy achieved a live birth after the first complete cycle [105]. Additionally, another study indicated positive results in poor ovarian responders, and suggested the freeze-all strategy as an alternative to cycle cancellation for these patients [110]. Despite these positive results, large multi-centre randomised controlled trials are needed to evaluate the freeze-all strategy [105, 110].

4. Conclusion

In conclusion, the cryopreservation of gametes and embryos is a rapidly developing field that demonstrates increasingly comparable success rates to those encountered in conventional IVF using fresh gametes or embryos. Aiming to provide reproductive autonomy for patients, it is intrinsically intertwined with both societal and ethical issues, and will doubtless play an increasingly central role in how we as a species reproduce over the coming decades. Research indicates also safety of reproductive treatments using cryopreserved gametes and embryos.

Acknowledgements

K. Rodriguez-Wallberg is supported by research grants from Stockholm County Council, Karolinska Institutet, The Swedish Cancer Society and Radiumhemmets's Research Funds.

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