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Review

Ice age: Cryopreservation in assisted reproduction – An update

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ABSTRACT

Since the first reported birth following in vitro fertilization in 1978, further developments in assisted reproductive technology (ART) treatments have produced at least 8 million babies worldwide. Cryopreservation techniques have been central to this treatment revolution, increasing cycle efficacy by allowing the banking of supernumerary embryos for later use, as well as affording the prospective patient more time in cases of anticipated fertility decline. Additionally, these techniques have demonstrated promise in increasing the safety of ART treatments, by reducing complications such as ovarian hyperstimulation, leading to increased support for the introduction of a 'total freeze' policy involving deferred embryo transfers. Importantly, the effective cryopreservation of both spermatozoa and oocytes has permitted long-term gamete storage without degradation of quality, facilitating gamete banking for personal use or fertility treatment. Here, we will summarise the indications for applying cryopreservation methods in clinical reproductive medicine, highlighting recent technical advances and examining the evidence base that supports the continued use of cryopreservation in ART.

1. Introduction

Remarkable advances in clinical and laboratory practice over the past number of decades have produced dramatic changes in what is achievable in the context of fertility medicine, affording options that were previously impossible [1]. The advent of safe, reliable cryopreservation protocols has facilitated the widespread use of gametes and embryos that have been frozen and subsequently thawed in assisted reproduction technologies (ART). Historically, semen cryopreservation was achieved first, as a direct consequence of the large number of potentially viable spermatozoa obtainable per sample. As oocytes are both more prized and more fragile gametes, it took many years for cryopreservation techniques to evolve sufficiently for their efficient suspension, making embryo cryopreservation the traditional first-choice technique for fertility preservation in adult women. More recently, improvements in gamete cryopreservation have allowed for increased female reproductive autonomy, but are not without their respective drawbacks. This review will therefore provide updated information on practical and technical aspects of cryopreservation technologies in the clinic, as well as ethical perspectives and future considerations. Here, we have chosen to discuss the cryopreservation of spermatozoa, embryos and oocytes separately and sequentially, so as to avoid confusion and to reflect their development chronologically.

2. Clinical applications of cryopreservation

Cryopreservation has allowed gametes and embryos to be suspended in time, enabling clinical practice to advance through the provision of time and flexibility. It has resultantly facilitated the development of gamete banking (for medial or social reasons), gamete and embryo donation programmes and the provision and storage of reproductive materials for research. Its use cases will be discussed below (Table 1).

2.1. Cryostorage for fertility preservation

The parallel development of multiple avenues of ART has facilitated the integration of cryopreservation as a vital tool in several areas of clinical practice, allowing the prolonged conservation (and effective revitalization) of human cells required for the later production of progeny. Logically, therefore, the utility of cryopreservation techniques is apparent in cases where either male or female patients anticipate an imminent decline in gamete viability (and therefore, fertility) as a consequence of medical or iatrogenic causes (non-elective), or in cases where advanced maternal age is a growing fertility concern (elective/'social'). In women, conditions indicating non-elective preservation encompass both those that directly and indirectly cause premature

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Table 1
List of clinical indications for cryopreservation of sperm, oocytes and embryos.

Sperm	Oocytes	Embryos
Fertility preservation	Fertility preservation	Preservation of supernumerary embryos
Avoid repeated testicular surgery	Delay transfer	Deferred embryo transfer
Future fertility treatment	PCOS	Fertility preservation
Sperm donor programmes	Oocyte donor programmes	Embryo donation programmes
	Ethical concerns: avoid freezing multiple embryos	

PCOS – polycystic ovary syndrome.

ovarian insufficiency (POI). Primary ovarian conditions include severe ovarian endometriosis, bilateral benign ovarian tumours and certain genetic diseases (Turner's syndrome, Fragile X syndrome), while secondary causes include any condition that requires the systemic administration of gonadotoxic chemo- or radiotherapy [2]. Included here are malignant diseases, which account for a high proportion of such cases due to the frequent need to administer potent compounds such as alkylating agents, shown to induce POI in 42% of women treated [2].

Although the intrinsically finite nature of female reproductive biology has led to a deservedly disproportionate focus on female infertility, it must be stressed that males are also markedly sensitive to gonadotoxic damage. In fact, up to 60% of male cancer survivors experience fertility disruption [3]. In addition, testicular cancer is the most common cancer in young men, is increasing in prevalence worldwide, and although the treatment is curative in most cases, it may result in permanent infertility after surgical or additional adjuvant treatments including chemo and radiotherapy. As such, improvements in curative cancer treatment rates, coupled with societal factors that promote the deferral of childbearing, have led to more patients who survive their disease but have yet to complete their families, increasing public awareness of such circumstances and demand for fertility-preserving procedures [4,5]. At present, although the cryopreservation of both gametes and embryos is considered effective, care of young cancer patients focuses upon the freezing of gametes (spermatozoa or oocytes), as such individuals are unlikely to have entered into a committed long-term relationship [5].

A related consequence of this technological advancement is the observable explosion in 'social' egg freezing, with the most recent report of the Human Fertilisation and Embryology Agency in the U.K., HFEA, indicating a more than two-fold increase between 2013 and 2016 [6]. Although many factors may influence a woman's desire to freeze her eggs for future use, lack of a partner and societal pressures to delay childbearing doubtless play a significant role, with many women feeling that they must prioritize the advancement of their career before aiming to have children. In this way, the cryopreservation of oocytes may afford patients increased reproductive autonomy, having the additional advantage over embryo cryopreservation that a male partner does not have to be selected. In this way, cryopreservation aims to ensure that fewer patients face the exceptionally difficult decision of later reproducing with a partner who may no longer be ideal, or not reproducing at all. The influence of marketing pressure from private enterprise on this must also be emphasised, however, with increased commercialisation driving demand and affecting public perception of both the success and necessity of social freezing treatments.

In contrast, fundamental differences in reproductive biology permit men to produce gametes well into advanced age, making social spermatozoa freezing extremely uncommon outside of sperm donation programmes. That said, there is increasing evidence to correlate increased paternal age at conception with increased risk of neurodevelopmental disorders and cancer development in their offspring [7–9]. Coupled with the well-established increased risk of gross chromosomal abnormalities in the progeny of women who conceive at an advanced age, this adds to mounting evidence that gametes collected from younger individuals may produce 'healthier' offspring.

2.2. Cryopreservation adding flexibility to clinical practice

Another use of cryopreservation is its implementation as a clinical tool to refine certain aspects of assisted reproduction techniques. Historically, when advancements in ovarian stimulation protocols increased the yield of mature oocytes retrieved per cycle, the result was a corresponding increase in the number of embryos obtained that were eligible for replacement. As the high proportion of multiple births associated with ART has been a longstanding recognized limitation, there was a consequent need to cryopreserve the remaining untransferred embryos following the recommended practice of a single 'fresh' embryo transfer [2]. As such, the clinical introduction of cryopreservation in the treatment protocols of couples that do not necessarily require it has led to a global reduction in the number of multiple pregnancies produced as an unwanted consequence. This process of transferring a single fresh embryo initially, followed by additional rounds of frozen embryo transfers using the supernumerary embryos has now become recommended clinical practice. Similarly, cryopreservation has facilitated the introduction of elective deferred embryo transfers as an option in clinical practice for patients who are at risk of, or present with, ovarian hyperstimulation syndrome (OHSS) and require time to recover, in cases which necessitate pre-implantation genetic diagnosis or screening (PGD/PGS), and where patients request the banking of supernumerary embryos for future use [1,10]. In addition, gamete cryopreservation may be used to increase the time available to patients to complete their treatment. As age is the factor that most significantly impacts cycle outcome, women of more advanced age may be offered multiple consecutive rounds of ovarian stimulation in order to cryopreserve a large number of eggs at as young an age as possible. These can later be fertilised and transferred, as necessary. This is advantageous in cases where multiple children are desired, or where the storage of large numbers of embryos is not ethically acceptable. Although this use case might be considered either medical or social, it is important that it is offered to eligible patients in either context.

2.3. Cryopreservation improving the Success of assisted reproduction

Cryopreservation techniques have greatly improved the safety and efficacy of IVF treatments, by allowing increased temporal flexibility and by permitting the deferred use of all viable embryos obtained from a single egg collection. To further improve ART outcomes and treatments, the implementation of a 'freeze all' strategy has been suggested, whereby all embryos created are cryopreserved, with deferred embryo transfer then carried out in the course of a natural cycle (or with exogenous oestradiol and progesterone replacement to 'prime' the endometrium). It is hypothesized that this strategy may result in the frozen-thawed embryos being re-introduced to a more 'physiological' milieu, seemingly correlating with improvements in implantation rates and outcomes compared to fresh transfer [10]. The evidence for this strategy will be further discussed below.

3. Cryopreservation of spermatozoa

Sperm cryopreservation was first introduced in the 1950s, following the fortuitous discovery that glycerol protected such cells from

cryopreservation-related damage. Their small size and relative abundance for experimentation facilitated the development of effective freezing protocols ahead of larger structures such as embryos and oocytes. This property is therefore a very important one, with smaller structures proving less susceptible to damaging ice-crystal formation during the cryopreservative process. Slow-freezing protocols were the first method used to successfully freeze spermatozoa, and remain the most commonly used technique worldwide [11]. Increasing scrutiny has led to the discovery that slow-freezing protocols may induce significant genetic damage, with this constellation of damaging factors explaining the low post-thaw mobility rate traditionally observed (60%) [12]. As such, this evidence and a need to cater for patients with extremely low concentrations of spermatozoa led to renewed research efforts, producing a number of distinct preservation techniques, including rapid freezing (vitrification). Although these techniques had been employed successfully elsewhere (initially with embryos and, in the last decade, with oocytes), the generally high numbers of spermatozoa obtained per sample made lower survival rates acceptable.

3.1. Techniques for the cryopreservation of spermatozoa

Traditionally, slow-freezing and vitrification protocols were considered comparably acceptable in the clinical setting. More recently, comparative studies have demonstrated that vitrification methods produce modest improvements in post-thaw motility rates and produce less disturbance in protein expression profiles, as well as being more cost and time-efficient [13,14]. Moreover, more recent refinements in vitrification protocols specific to spermatozoa have eliminated the need for cryoprotectants, improving post-thaw vitality, motility and acrosomal preservation, as well as reducing the incidence of DNA fragmentation [15]. As such, it is possible that the real strength of vitrification may lie in the ability to eliminate cryoprotectant-mediated (osmotic) damage. Further gains in efficacy may result from the implementation of cryoadditives; with the antioxidant querctin, catalase and brain-derived neurotrophic factor (BDNF) having been shown to produce improvements in post-thaw viability, motility and DNA damage [16–18].

Additional variations in cryopreservation protocols exist in terms of the methods used to store samples once they are cryopreserved as well as the methods of sperm selection, in particular when the sperm titres are reduced. A list of methods for sperm collection, selection and storage is presented in Table 2.

Reassuringly, it has been reported that long-time storage (40 years) does not appear to affect the post-thaw fertilization potential of sperm [19].

Generally, therefore, current research efforts are focused on refining cryopreservation protocols in order to minimise DNA or membrane damage that could later impact fertilisation (and therefore clinical pregnancy) rates, on improving storage methods and conditions, and on both generalized and precise selection methods aimed at ensuring that the spermatozoa chosen for use in fertilisation are best available.

3.2. Sample collection and service uptake

In most cases, the sperm samples required for cryopreservation are obtained via masturbation. Rare exceptions include cases of obstructive azoospermia, instances where patients are unable to produce a sample (e.g. for physical or psychosocial reasons), or where a vasectomy has previously been carried out, surgical techniques may be employed (Table 2). Importantly for oncological applications, it has been shown that no correlation exists between sperm quality and disease stage, meaning that all patients should continue to be considered for cryopreservation of sperm through semen banking [20]. Options are greatly reduced for patients with non-functional Sertoli cells, or pre-pubertal males, however. It is envisaged that the cryopreservation and post-treatment autotransplantation of spermatogonial stem cells (SSCs) may be a possible future treatment, having shown promise in experimental animal models but not yet introduced to human trials [21].

Even though most guidelines recommend that the option to cryopreserve spermatozoa should be discussed with all post-pubertal males prior to initiation of gonadotoxic treatment, evidence demonstrates that only a quarter of eligible males opt to avail of such a service [22]. Given the robust evidence base that demonstrates that almost all men diagnosed with cancer wish to have children later in life, and the non-invasive nature of semen sample collection, these numbers are unexpectedly low [22]. The potential causes of this lack of uptake may include inadequate counselling by clinicians or unfounded concerns about malignant transmission to progeny. Equally, it is possible that young male patients may not prioritise their reproductive potential to the same extent as their female counterparts, or that male stoicism may affect individuals' ability to discuss their future aspirations, thereby impacting the quality of the decisions made. The perceived high cost of sperm cryopreservation and storage might also have a role to play in many countries, despite cost-benefit analyses demonstrating it to be a more cost-effective option than delaying fertility treatment until treatment has ended [23].

3.3. Clinical outcomes of using cryopreserved spermatozoa

'Success' in reproductive medicine can be calculated in numerous different ways, depending on the perspective of the individual concerned. For most patients, however, the most relevant statistics are those relating to live birth rates and perinatal outcomes. As such, data from robust meta-analyses indicate that there is no statistical difference in clinical pregnancy or fertilisation rates in couples undergoing ICSI or IVF using cryopreserved spermatozoa, when compared to fresh samples [24]. These results were echoed by more recent analysis by Ferrari and colleagues, and data from a large Australian sperm donation

Table 2
Individual methods for sperm collection, selection of sperm for cryopreservation and storage.

Collection Methods	Selection Options	Storage Options	
		Biological carriers	Non-Biological carriers
Masturbation	Pre-freeze swim-up	Empty zona pellucida	Straws
TESE	Density gradient	Volvox globulator spheres	ICSI Pipette
Micro-TESE	MACS		Cryoloop
TESA	Zeta Potential Selection		Microdroplet
MESA	Electrophoresis		
PESA			
Open epididymal fine-needle aspiration			
Microsurgical testicular sperm extraction			

TESE - Testicular Sperm Extraction; TESA - Testicular Sperm Aspiration; MESA - Micro-Surgical Epididymal Sperm Aspiration; PESA - Percutaneous Epididymal Sperm Extraction; MACS - Magnetic Activated Cell Sorting/ ICSI – Intracytoplasmic Sperm Injection.

programme, which also demonstrated no statistically significant increase in adverse perinatal outcomes, congenital or chromosomal abnormalities when cryopreserved samples were compared to fresh [25,26].

In light of the previously discussed evidence that cryopreservation damages spermatozoa, the similarities in success rates observed are interesting. It is possible that this is a logical consequence of the large number of sperm usually contained within each sample, driving interest in whether these rates might differ in patients with reduced sperm titres. As such, there have been increased efforts to elucidate the impact that certain forms of spermatozoal damage (e.g. DNA fragmentation) might have on treatment outcomes, with the aims of informing methods of sperm selection for fertilisation, and consequently of guiding management of azoo- or oligospermic patients. The lack of correlation of DNA damage burden with clinical pregnancy rates achieved may be explained by work by Meseguer et al., who determined that the relative impact of sperm DNA fragmentation on pregnancy outcome was dependent on the quality of the oocyte available for fertilisation [27]. In other words, if spermatozoal DNA has sustained damage, then it may be possible for this damage to be repaired by the DNA repair machinery of the oocyte, but if the oocyte itself is of poor quality, this is less likely to occur. Therefore, although parameters of sperm quality such as motility and DNA fragmentation may be of some importance in andrological work-up, the quality of the oocytes obtainable for use is much more significant in determining cycle outcome.

4. Embryo cryopreservation

Since the first reports of a successful pregnancy following embryo cryopreservation emerged in 1983, more than half a million live births have been achieved through such methods [28]. Occurring more than 30 years after the clinical introduction of spermatozoa cryopreservation, it represented a quantum leap forward in ART, and has gone on to improve options, efficacy and safety for prospective patients. In their last report, the European Society of Human Reproduction and Embryology (ESHRE) revealed that 192,017 frozen-thawed embryo transfer (FET) cycles were performed in Europe in 2014. This represented a 24.1% increase over the same period the previous year, exceeding for the first time the number of IVF treatments, and evidencing the increasingly central role that embryo cryopreservation is playing in clinical practice [29]. In fact, some countries such as Switzerland, the Netherlands, Norway, Finland, Czech-Republic, Iceland, Belgium, Poland and Sweden report the proportion of cryopreserved embryo transfers performed as being greater than 50% of all embryo transfers, although regulatory stipulations may have a role to play in such cases [29]. Irrespective of such regulations, the use of embryo cryopreservation will continue to increase due to novel indications, such as the "freeze-all" strategy to reduce complications and pre-implantation genetic screening at the blastocyst stage.

4.1. The expanding role of embryo cryopreservation in clinical practice

Unwanted multiple births have historically been a source of much consternation in ART. Prior to the introduction of embryo cryopreservation, no methods of conserving supernumerary embryos for future use existed, meaning that clinicians encountered pressure to replace > 1 embryo at a time in order to increase the likelihood of cycle success. As such, cryopreservation facilitated the development of frozen-thawed embryo transfer (FET) techniques, having the dual effect of increasing pregnancy rate per stimulation cycle and of decreasing multiple birth rates. As the technique became more established, its applications expanded outside of improving IVF cycle efficacy to encompass the preservation of fertility in at-risk patient subgroups and social embryo banking. Furthermore, it has permitted deferred embryo transfer, in cases of OHSS, for example. An interesting consequence of the use of cryopreservation in this context has been the observation of improvements in clinical pregnancy rates when cryopreserved embryos are used rather than fresh samples [10]. In addition, fresh embryo transfer has been associated with poorer obstetric and perinatal outcomes when compared to FET [30]. It is hypothesized that these observations may be the consequence of the supraphysiologic hormone levels that occur during controlled ovarian stimulation, which are thought to negatively impact endometrial modification. This hypothesis is supported by analysis showing that these differences are accentuated in patients who demonstrate high ovarian response [31]. Until recently, there were insufficient data in patients who exhibit poor ovarian response to recommend a total-freeze strategy for the general IVF population, with clear benefit only demonstrated in cases of polycystic ovary syndrome (PCOS), OHSS, high-ovarian responders and recurrent implantation failure [32]. However, Zhu et al. have since reported a 50.74% LBR per stimulation cycle in a general IVF cohort [34], while another study detailed promising results in patients demonstrating poor ovarian response [33,34]. To put this in perspective, a prospective study of 156947 women in the UK found the LBR achieved with conventional IVF strategies to be 29.5% [35]. The authors of the latter study went on to suggest that another application of the freeze-all strategy might be as an alternative to cycle cancellation for these patients [33,34]. Although this initial evidence from retrospective studies is promising, large multicentre RCTs are needed to demonstrate superiority of the freeze-all strategy sufficiently to warrant its widespread clinical introduction. Such trials would have to duly consider the increased risk of preeclampsia for those assigned to a freeze-all cohort [36].

4.2. Methods of embryo cryopreservation, culture and transfer

As with spermatozoa, methods and protocols for the cryopreservation of embryos have been refined over the course of their clinical use, leading to improvements in embryo survival post-thaw and live birth rates. As large, multicellular structures, embryos are more resistant to freeze-thaw damage, and therefore early attempts to preserve them at −196 °C using slow freezing were largely successful. In slow-freezing protocols the temperature is gradually decreased at a controlled rate after pre-equilibration with cryoprotectants to reduce intracellular ice formation. Although this method has been used safely and extensively in IVF laboratories across the world, it does not entirely avoid ice crystal formation and its associated damage. Therefore, there was a need to develop a newer technique, vitrification, which has become favoured over the last decade [37,38].

Vitrification achieves an extremely high cooling rate through exposure to liquid nitrogen, and produces a glass-like state with vastly reduced ice crystal formation [39]. Despite requiring extremely high concentrations of cryoprotectant, the toxicity of this technique is contained at an acceptable level through the use of small volumes and short exposure times [38]. With vitrification, a post-thaw embryo viability between 78% and 100% is usual, comparing favorably to the 60% obtained with the slow-freezing method, and correlating with an improvement of the clinical pregnancy and LBR per embryo transfer [37,38,40]. In addition, it has the advantages of not requiring specialised or expensive equipment and being less time-intensive. When these factors are taken into account, the widespread adoption of vitrification in place of slow-freezing seems logical [38].

Although these are the two main freezing techniques used, protocols vary in terms of the cryoprotectants used (concentration and type), equilibration timing, cooling rates and freezing devices (Table 3). Another possible procedural variation is the stage of development at which embryos are cryopreserved. Current practice involves the preservation of embryos at either the cleavage stage (Day 2 or 3 of culture) or at the blastocyst stage (day 5 or 6). There is currently no clear evidence to suggest the superiority of either strategy for frozen-thawed embryo transfer, although it has been suggested that some specific patient subgroups might benefit from transfer at the blastocyst stage [41–43]. It

Table 3
Morphology parameters for embryos at time of cryopreservation and criteria for embryo transfer after thawing with associated cryopreservation protocols [37,42].

	Cleavage stage		Blastocyst stage	
	Day 2	Day 3	Day 5	Day 6
Morphology before cryopreservation	≥4 blastomeres < 25% fragmentation No multinucleate blastomeres	≥6 blastomeres	Blastocyst quality are scored according to expansion, inner mass and trophectoderm using Gardner scoring system – 3BB or higher	
Morphology after thawing	≥50% intact blastomeres Higher number of blastomeres after 24 h of culture		Same classification as before cryopreservation	
Cryopreservation protocol	Slow-freezing (PrOH + Sucrose) Vitrification (EG/DMSO/sucrose)		Slow-freezing (Glycerol + Sucrose)	

PrOH – 1,2-propanediol/EG - ethylene glycol /DMSO – dimethylsulphoxide.

is important to stress that much of the available data concerning the timing of embryo cryopreservation is derived from outcomes of fresh cycles, as few studies have combined a 'freeze-all' approach with comparative analysis of the developmental stage at which the embryos are preserved [44]. Encouragingly, the length of time spent in storage does not appear to influence post-thaw viability or pregnancy outcome [45].

4.3. Clinical outcomes in embryo cryopreservation

The increasing number of frozen-thawed embryo cycles being performed has led to heightened concerns about any possible impact that cryopreservation may have on perinatal outcomes [1]. Although embryo cryopreservation is a well-established procedure, long-term studies have traditionally been lacking [38]. Most were cohort studies (unmatched cohorts) with little information obtained from RCTs. One year ago, however, Maheshwari et al. published a systematic review with cumulative meta-analysis exploring the clinical outcomes of using cryopreserved embryos [46]. Reassuringly, the data suggested that pregnancies obtained from frozen embryos are not associated with increased perinatal risk when compared to fresh transfers. Results even indicated that the use of cryopreserved embryos conferred a decreased risk of offspring having low birth weights (RR 0.61 for prematurity; 0.72 and 0.76 of having babies weighing less than 2500 and 1500 g respectively). In fact, FET seems to increase the risk of having bigger (RR 1.54) and heavier babies (RR 1.95 for > 4Kg; RR 1.86 for > 4.5 Kg). It must be stressed, however, that all of the studies included in this analysis (bar one) were observational and not randomized. Thus, the finding of macrosomia and large babies for their gestational age after FET deserve further follow-up studies [47]. Another interesting observation has been the association of fewer observed ectopic pregnancies with FET when compared to fresh cycles, with even fewer ectopic pregnancies being observed in blastocyst stage transfers (especially day 6) [48,49]. These figures aside, it is important to note that both approaches are inferior to spontaneous conceptions in terms of perinatal and neonatal risk [50].

As discussed above, the cryopreservation process does not appear to negatively impact clinical pregnancy or LBR, and may indeed prove superior to fresh transfer in this respect, although further studies are required to validate this approach. Other future avenues of research interest include the use of letrozole as an alternative to HRT or natural cycles in patients undergoing FET; a strategy that improved LBR and reduced miscarriage rates in one Japanese study [51]. While this was a retrospective cohort study, and therefore potentially subject to selection bias in terms of the patients who were selected for letrozole treatment, it is an approach that warrants further (prospective) analysis.

5. Cryopreservation of oocytes

Despite its technical difficulty, the cryopreservation of oocytes was a necessary developmental step in the provision of ethical and effective ART. As a consequence of their large size, oocytes retain more water and are therefore more likely to sustain crystallisation-mediated damage. Furthermore, their relative scarcity necessitates a minimization of freeze-thaw damage and a maximization of attrition rates. Understandably, therefore, much work was required to refine the protocols used, with the first efforts marred by zona pellucida thickening, premature cortical granule exocytosis and disturbance of the meiotic spindle [52,53]. Although this latter issue is almost always transient, there is recent evidence to suggest that cryopreservation may have more subtle, previously undetected effects on oocyte gene expression and proteomics. Whilst these studies are far from conclusive, the observed alteration of maternally-derived proteins which support early embryo development warrants further investigation [54,55].

5.1. The advent of vitrification and its path to the clinic

Combined, the factors discussed produce an oocyte that is resistant to penetration by spermatozoa. As this obviously hinders the fertilisation process, it proved a long-standing stumbling block that limited the clinical implementation of cryopreservation until the arrival of ICSI. The first successful pregnancy in 1997 demonstrated the validity of the technique, paving the way for a series of leaps forwards in procedural technique, culminating with the most significant; vitrification [56]. Indeed, it was only with the inception of vitrification that post-thaw oocyte parameters attained standards compatible with widespread clinical introduction. This was highlighted by a 2014 Cochrane review, which reported that vitrification increased oocyte survival by 29%, and fertilisation by 19%, when compared to slow-freezing [57]. These results were further validated by a large prospective study of Spanish egg donation programs, which failed to demonstrate any difference between fresh and vitrified oocytes across a multiparametric analysis [58]. Although these data are encouraging, it must also be remembered that the vitrification process is an extremely quick one, making it especially variable and operator-dependent. Therefore, the results above may not be reflective of results obtained in many IVF clinics. Another important consideration is the age of the donor in question and the stage of malignancy in those afflicted, as both of these factors have been shown to impact the post-thaw survival of vitrified oocytes [59]. As such, the high survival rates (> 96%) reported in some egg donation cohorts should be interpreted with caution, especially when coupled with the impact that the aforementioned factors also has upon the number of eggs collected.

Although the above evidence demonstrates clear superiority of vitrification in terms of post-thaw survival, it was not known until relatively recently whether or not this corresponded with an increase in the pregnancy rate achieved. This question was addressed by a number of groups, who reassuringly found the clinical pregnancy rate (CPR) to increase by more than 100% when vitrification techniques were employed [60,61].

In the quest to uncover further avenues of potential improvement, thorough examination of other variables in experimental procedure has been carried out. Of these, one of the most promising has been the warming rate implemented during the thawing process. Initially cited as a potential source of refinement by Mazur and Seki (who achieved oocyte survival > 80% even when used with traditional slow-freeze protocols), warming cryopreserved oocytes at an 'ultra-rapid' rate has more recently been applied to vitrification. While the post-thaw survival of vitrified oocytes is already extremely high, advances such as this allow the stringency of other protocol parameters, such as permitting the use of reduced concentrations of cryoprotectant, thereby

reducing the toxicity incurred [62].

5.2. Clinical outcomes in the cryopreservation of oocytes

Although vitrification has produced impressive post-thaw statistics such as the > 96% reported above, figures like this do not accurately portray the realities faced by patients clinically. If quoted in such terms, it would be understandable that lay-people might believe their chances of achieving a pregnancy to be extremely high, leading to poor financial planning or increased willingness to delay childbearing with the belief that their cryopreserved eggs are a fool-proof insurance policy. This is an especially important consideration in the current climate of increasingly commercialised fertility treatment, where private clinics may partially obfuscate discouraging statistics in favour of increasing the number of cryopreservation procedures that they carry out. Therefore, the most realistic (and ethical) way to present the likelihood of 'success' using cryopreservation techniques is using live birth rate per oocyte thawed. While this might seem obvious, many clinics opt instead to quote clinical pregnancy rates per thaw cycle; a figure that does not take into account variations in the number of eggs a patient may have available to thaw, and therefore may not accurately represent the reality faced by most patients. Illustrating this, the largest reported study of 3610 vitrified oocytes reported an oocyte survival rate of 90%, translating to a clinical pregnancy rate of 48% and an 'oocyte to baby' rate of just 6.5% [63]. Were this same study to be presented differently, this would correspond to a delivery rate of 78.8% per donation cycle, highlighting the potential for misrepresentation or misinterpretation. It is also important to emphasise that this study used donor oocytes; eggs which tend to be from carefully selected younger patients, and therefore of higher quality. As such, it is likely that the true likelihood of 'success' for the average patient undertaking oocyte cryopreservation is significantly lower than the figure quoted above. In light of this, increased efforts must be made to homogenise how success is presented, and to investigate the true 'oocyte to baby' rate in the general autologous cryopreservation cycle population. Cobo et al. produced the most reliable data in such a cohort, evaluating the reproductive success of 1468 women who undertook elective cryopreservation for non-oncologic reasons [64]. Although unfortunately omitting an 'oocyte to baby' ratio, their data are informative, and highlight the impact of age on success rates, quoting a 53.9% live birth rate per ET if the oocytes used were cryopreserved before the age of 35, but a 22.9% live birth rate per ET if the same procedure was carried out after the age of 36. Whilst the importance of age in determining treatment outcome in fertility treatment in general is generally appreciated, these figures may help clinicians to illustrate this in the context of cryopreservation. Following on from this, a 2018 HFEA report cited age at freezing to be 'the most important factor', while finding that age at thaw (and use) did not have a statistically significant impact [6]. The same study also re-emphasised the importance of presenting success in the context of the number of oocytes thawed, finding that an increase from 5 to 8 available oocytes translated to a 25.4% increase in live birth rate, with every additional oocyte producing an 8.4% increase (if patient < 35).

Although the success rates outlined above give a general representation of the situation faced by patients, evaluation of the impact of individual variables upon these figures is also necessary. Perhaps the most obvious of these, the cryopreservative method employed, was analysed by a recent comparative meta-analysis, which concluded that oocytes preserved using vitrification techniques produced significantly higher fertilisation and embryo cleavage rates, along with increasing the proportion of 'top-quality' embryos produced [65]. A limitation of this study, however, was the disparity between the vitrification and slow-freezing groups in terms of the number of oocytes frozen, with only 361 slow-frozen oocytes included, compared to 4282 vitrified oocytes. Importantly, this analysis also included 3524 fresh oocytes, and found that "the rates of ongoing pregnancy, top-quality embryo, embryo cleavage and fertilisation did not differ between the vitrification and the fresh oocyte groups". As such, it is possible that cryopreservation techniques have advanced to a stage where thawed oocytes near equivalency with fresh oocytes. If true, the significance of this is that major improvements in the 'success' of these techniques is likely to come from methods of increasing the yield of oocytes collected per stimulation cycle, or in methods used to select which specific oocyte of a number in culture is most likely to produce a pregnancy, and should therefore be transferred. One such example of this is the recent finding that pre-treatment with the antioxidant coenzyme Q10 in low-prognosis young women with decreased ovarian reserve may improve ovarian response to stimulation and increase both the number of oocytes retrieved and the fertilisation rates observed [66]. Reassuringly, the length of cryostorage does not appear to impact pregnancy rates, having been investigated by a number of research groups [63,67]. In summary, it is likely that egg-freezing methods are on-course to produce thawed eggs of similar reproductive potential to fresh eggs, however a certain (small) proportion of oocytes will not survive the freeze-thaw process, and the chance of producing a live birth *per oocyte cryopreserved* remains low. This should be communicated to patients to ensure transparency and avoidance of over-reliance on preserved eggs for family planning purposes.

Another essential aspect of any discussion on clinical outcomes is that of perinatal outcome. While clinical pregnancy rate is an important comparator when discussing treatment, consideration must also be given to whether or not the pregnancy (and offspring) produced are 'normal'; i.e. morphologically, genetically and developmentally. Reassuringly, several analyses (one of 165 pregnancies and another of 936 infants) failed to find any increase in adverse outcome in pregnancies originating from vitrified oocytes, or any increase in the incidence of congenital abnormalities observed. In fact, the latter study determined that the incidence of congenital abnormality to be comparable across infants born following oocyte vitrification, conventional 'fresh' IVF and natural pregnancy [68,69]. These data are even more reassuring when it is considered that the average age of women conceiving using ART is higher than the general population, with increased age having already been reliably associated with adverse perinatal outcome. As such, it seems unlikely that a cryopreservation-specific (or indeed ART-specific) causal relationship will ever be reliably established.

In conclusion, oocyte cryopreservation technologies have advanced dramatically over the last decade, facilitating effective treatment without increasing risk of adverse outcome. That said, the oocyte-to-baby conversion ratio remains low, and should be quoted to the prospective patient as part of ethical clinical practice. It must again be emphasised that modern ART circumvents the physiological mechanisms that select for the most viable gametes in vivo; as such, development of novel selection methods might allow for continued improvement of the success rates observed across ART, in both fresh and cryopreserved cycles.

6. Cryopreservation – legal, societal and ethical perspectives

The development of clinically-reliable methods, such as the vitrification of oocytes for women, continues to influence the practice of fertility preservation in those with oncologic or benign indications. As the options of preserving embryos or oocytes promise to become similarly successful, even more choices will be afforded to women in need, allowing them to make a rapid decision about future fertility possibilities. A recently-reported large prospective study (spanning 20 years in Sweden), indicated that the such women increasingly elect to freeze their oocytes unfertilized, instead of freezing of embryos, even if they are in a committed relathioship and have a partner [70]. This may be explained by the legal constraints regarding embryos in some countries, as the use of cryopreserved embryos may not be later allowed if the couples have split, but also indicates an increased awareness of female reproductive autonomy in the future [70].

Given the increasing demand for gamete cryopreservation, especially in the social context, it is important that we consider the potential ramifications of such procedures. Firstly, in an age where genetic material is increasingly interpretable, and increasing emphasis is put on the safety of such data, it is likely that such factors will necessitate the parallel development of increasingly more stringent security protocols concerning how gametes are cryopreserved, handled, stored and thawed. Such measures will protect the wellbeing of prospective children and ensure that genetic information is not used for purposes other than those consented to by donors. For example, the new General Data Protection Regulation (GDPR) has introduced punitive consequences for any individual or business found to be misusing genetic data [71].

Societally, the use of cryopreservation allows women to defer childbearing of genetically-related offspring to a stage that might otherwise be considered unwise. Although this may benefit individuals in terms of career progression, it also furthers the commercialisation of ART and the advertisement of cryopreservation techniques as an insurance policy against age-related fertility decline. This is most notably seen in the increase in the number of companies offering 'social freezing' as part of their employee benefit packages. While it is likely that this comes from a beneficent place in a majority of cases, it serves to perpetuate the societal pressure placed on women to delay childbearing. A connected issue is the expense of such procedures. Usually, there is an up-front cost involved with the initial collection and cryopreservation of eggs, followed by a yearly storage cost. As such, the cost-effectiveness of such procedures depends on the age at which freezing is carried out, and the age at which the gametes are utilised. Understandably therefore, cost-benefit analyses have provided contrasting conclusions on whether it is better to freeze one's eggs at a young age and use them later on, or just to attempt conventional IVF earlier [72,73].

Finally, it is possible that the future of cryopreservation may involve the preservation of gametes at a young age in order to minimise the risk of age-related genetic abnormalities. Cases of such widespread use would necessitate an increase in the amount of longitudinal data available, in order to exclude potential knock-on effects that may not be immediately obvious. As such, as reproductive technology advances, increased discussion on what should and should not be permitted is essential.

7. Conclusion

In conclusion, the cryopreservation of embryos and gametes is a rapidly developing technology with success rates that are increasingly comparable to those achieved using fresh gametes. The widespread application of cryopreservation, both in the lab and the clinic, has already had a lasting effect on the way that assisted reproduction is performed, and has allowed patients increased flexibility in terms of when and how they reproduce. Given the increasing societal pressure to delay childbearing, its role in the future is only likely to increase. As such, it is hoped that this review provides a snapshot of the evidence that has shaped its development and establishment as a safe and effective adjunct to reproductive treatment.

Author contribution

All the authors contributed to the writing of the manuscript, and all authors approved the final version.

Conflict of interest

The authors have nothing to disclose

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