CryoLetters 46(4), 213 – 230 (2025)

https://doi.org/10.54680/fr25410110112

PERSPECTIVE

ADVANCEMENTS IN CRYOPRESERVATION TECHNIQUES FOR HUMAN GAMETES AND EMBRYOS: NOVEL CRYOPROTECTANTS AND THEIR INFLUENCE ON FERTILITY PRESERVATION

A. S. Vickram*, C. Prasanth, S. Bharath, D. Jenila Rani, C. M. Mathan Muthu and B. Bhavani Sowndharya

Department of Biotechnology, Saveetha School of Engineering, SIMATS, Chennai, 602105, India
*Corresponding author's E-mail: vickramas.sse@saveetha.com

Abstract

BACKGROUND: Cryopreservation has transformed fertility preservation by enabling the long-term storage of gametes and embryos. Although traditional procedures such as slow freezing and vitrification are routinely used, recent innovations and new cryoprotectants have had a significant impact on reproductive medicine outcomes. **OBJECTIVE:** This review examines advances in cryopreservation techniques, the influence of novel cryoprotectants, and the implications for gamete and embryo survival, viability, and clinical outcomes in fertility preservation. **MATERIALS AND METHODS:** We conducted a comprehensive review of the existing literature on classic and novel cryopreservation methods for sperm, oocytes, and embryos. Advances in vitrification methods, the invention of novel cryoprotectants, and comparative effectiveness and toxicity assessments were all evaluated. Clinical data on survival rates, implantation rates, and fertility preservation were thoroughly reviewed. **RESULTS**: Improvements in vitrification procedures have drastically enhanced oocyte survival and developmental potential, resolving some of the previously linked cryopreservation issues. Innovative ways to cryopreserve have enhanced sperm survival and motility after thawing. The focus of embryo cryopreservation has switched from traditional slow freezing to precise vitrification, resulting in higher survival rates and better clinical results. Novel cryoprotectants have shown promise in terms of reduced toxicity and improved cryosurvival while retaining biological integrity. Overall, these advances have had a positive impact on fertility preservation techniques and clinical success rates. **CONCLUSION**: Emerging cryopreservation methods, such as breakthroughs in vitrification protocols and the identification of new cryoprotectants, have significantly improved gamete and embryo storage efficiency. Such developments not only increase the longevity and quality of cryopreserved materials, but they also improve therapeutic outcomes in fertility preservation. Additional study and optimization are required to standardize these procedures for optimal use in a variety of patient populations.

Keywords: cryopreservation advancements; embryo cryopreservation; ethical cryopreservation practices; innovative cryoprotectants; sustainable biomedical practices.

INTRODUCTION

Reproductive medicine is currently significantly affecting our lives. Couples with poor reproductive ability are increasingly using its advantages to conceive and achieve their ambitions of having a family (1). A crucial way to make this element broadly accessible is the capacity to maintain fertility (sperms, eggs and embryos) via cryopreservation. The growing use of many cell types in clinical medicine, including stem cells, blood cells and human oocytes has made the necessity for cell cryopreservation an emergent clinical concern (2). Oocyte cryopreservation has thus become a viable option to preserve female fertility and in assisted reproductive medicine.

Cryopreservation is a technique of sustaining cellular life that involves freezing at very low temperatures. A cryoprotective agent is employed throughout the cryopreservation procedure to safeguard cellular structures from harm during the cryogenic and rewarming processes (3). These developments should make it possible to offer long-term oocyte cryopreservation as a viable option for preserving fertility to women who have or are at risk of developing pathological conditions (such as tumour, cysts, and premature ovarian failure) or who may experience gonadotoxic effects after undergoing radiation therapy, chemotherapy, or other ovarian-damaging treatments (4). Lastly, oocyte cryopreservation provides an alternative to the ethical and legal concerns associated with embryo freezing (5).

Slow freezing of oocytes with in vitro fertilization yields low rates of childbirth (13%). This consequence has been related to irreparable harm to cryopreserved oocytes, the potential of chromosomal misalignment and hardening of the zona during gradual freezing (6, 7) lose-pulled straw, Cryoloop (metal loop used to hold a small drop of cryoprotectant) and Cryotop (plastic strip with a small tip that holds the biological sample) minimal volume vitrification methods were originally developed to treat the risk of damage during oocyte slow freezing (8).

The success achieved in semen cryopreservation has opened the door for notable advancements in the following fields: agriculture, mainly through the availability of an international exchange of germplasm of genetically improved animals; biotechnology, where scientifically relevant murine lines may be efficiently preserved by genomic resource banking, endangered animal cells conserved and human reproductive medicine supported (9). It is reasonable to concentrate on the physical changes that sperm go through during the entire process to improve the ease of sperm cryopreservation, since controlling these changes is undoubtedly likely to greatly increase survivability (10).

At the present time, traditional techniques for mammalian sperm cryopreservation are not ideal and produce variable effects according to the species, let alone the specific specimens within a single breed. The first birth of a newborn following the transplantation of a cryopreserved embryo was by use of progressive freezing in the conventional method (11). Much later, this method was applied successfully and very effectively for cleavage stage embryos and fertilized eggs or zygotes (12). However, higher usage of ARTs in humans including transfer of cryopreserved embryos necessitates enhancement of cryopreservation technology from the perspective of conception rates (13). As a result of the toxicity of CPA, there is always a search for the perfect non-toxic CPA to preserve cells at liquid nitrogen temperature (14). In the absence of cryoprotective agents (CPAs) in preservation solutions, the samples' survival and quality remain doubtful upon preservation. Nevertheless, the use of known conventional CPAs, such as DMSO, is limited especially in clinics due to its cell-membrane toxicity. The synthesis of high-efficiency and non-toxic CPAs remains a relevant challenge today. Polyols, sugars, amino acids, and Anti-Freeze Proteins (AFGPs) are examples of natural CPAs that are in charge of vital processes that allow cells to survive cold stress (16). AFGPs are biosynthesized by a range of species to survive in cold temperatures. These proteins have been used to enhance the survival of rat hearts in sub-zero non-freezing condition (17). Nevertheless, due to challenges in extraction and likely immunogenicity, their wider utilization has been limited. Existing research is directed at the synthesis of ice modulators such as phosphate buffered saline polyampholytes, oxidized quasi-carbon nitride quantum dots (to improve cell recovery from cryopreservation) and glycopolymers that can mimic the function of AFGP (18). These mimics have meaningfully positive effects on cell cryopreservation survivability. More study is required to

investigate the processes of AFGP involvement with ice formation, and to evaluate its efficiency for application in cryopreservation of vulnerable cell lineages, tissues and organs, and functional consequences such as apoptosis and differentiation ability of samples.

Cryoprotective agents reduce exposure to osmotic stress, stabilizing biomolecule and cell structure, and minimizing the effect of reactive oxidative species (ROS). The ideal CPA would neither osmotically dehydrate the cell or promote cryoinjury and it would be non-toxic (19). For instance, Figure 1 describes the molecular effects of cryopreservation, highlighting structural and functional changes in the sperm cell. The purpose of the cryoprotective agents should be to limit intracellular freezing, decrease cell damage due to the freezing environment, and enhance cell survival following thawing. High concentrations of CPAs have been demonstrated to contribute to increased cellular damage (20). In short there is a need for either improved CPAs for cell preservation or innovative, cost-effective cryoprotectants that are more reliable for conservation

SPERM CRYOPRESERVATION

Human sperm, which are easily obtainable, are currently the only cell type routinely preserved in germplasm banks (21). In 1953, the first documented attempt at achieving a clinical human live birth using frozen sperm was made through an early cryopreservation-assisted fertilization technique, which utilized semen stored at low temperatures in dry ice for short periods. Sperm freezing was made more popular in healthcare by 1963 through the discovery of long-term sperm preservation by means of liquid nitrogen (22). Sperm can degenerate during the freeze-thaw process due to osmotic and oxidative stress, cryoprotectant toxicity, and intracellular ice formation. Thus, it is necessary to regulate cryopreservation to reduce the likelihood of sperm damage, such as from ROS's detrimental effects on sperm function. Very slow cooling rates allow the cells to lose water to the extracellular solution, which may cause drying stress in the sperm. Conversely, if the cooling rate is fast, too little water is lost to extracellular ice and intracellular ice builds, which in turn deforms cell organelles (23). Neither of these outcomes is favourable for the survival of cells,

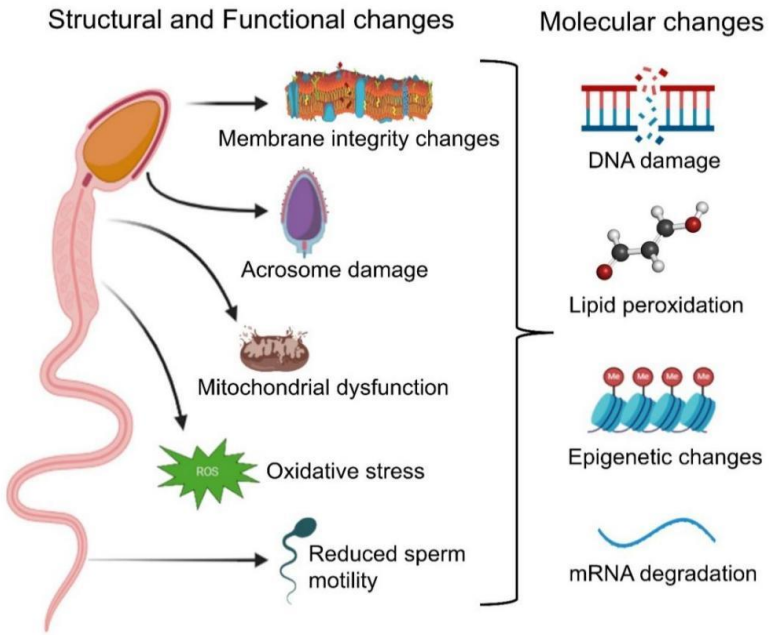

Figure 1. Molecular changes of sperm cells due to structural and functional changes induced by cryostorage processes. The diagram illustrates key structural and functional alterations in sperm, including membrane integrity changes, acrosome damage, mitochondrial dysfunction, and oxidative stress (ROS generation), ultimately leading to reduced sperm motility. These molecular changes are linked to DNA damage, lipid peroxidation, epigenetic modifications, and mRNA degradation, all of which compromise sperm function and fertility.

as both very low and very high cooling rates potentially lead to cell death. For successful cryopreservation, it is essential to identify the optimal cooling rate for the cellular system (24). Not only the cooling and warming rates are important, but also the choice and concentration of CPAs. For example, glycerol and egg yolk are known to reduce osmotic stress in spermatozoa. More complex CPA mixtures, incorporating additives such as zinc, resveratrol, and ascorbic acid have been used to enhance sperm preservation.

Traditional methods: slow freezing and vitrification

The conventional slow freezing method is the most widely used semen cryopreservation technique. The post thawing spermatozoa survival rate of cryopreserved samples is around 50%, whereas the variation of individuals is very large (25). However, slow freeze procedures have a number of issues since they require the right CPA and rely on a planned process of controlled chilling. According to some research, slow freezing significantly changes the sperm's gross morphology and functionality (26). Furthermore, ice crystals formed during sperm freezing may affect the sperm cytoskeleton, membrane and DNA. Sperm with DNA damage can be a significant contributor to male infertility (27).

Slow freezing can be achieved using a programmable cooler or nonprogrammable approach. It is time consuming as well as labour intensive. A gradual freezing method entails slow freezing of the sperm over a time span of around 2 to 4 h employing two or three changes in temperature either by hand or through a programmable freezer (28, 36). Primary cooling rates of the sample from room temperature to 5 °C have been found to be most effective at around 0.5 - 1 °C/min. On the other hand, vitrification refers to the procedure where cells are cooled at a very high rate to form a glass-like solid: it involves placing the sperm into liquid nitrogen vapour for 5 - 30 min before plunging it into liquid nitrogen (29). Previously, attempts made at vitrification of spermatozoa appeared unsuccessful, likely due to a suboptimal combination of permeable and non-permeable CPAs. On one side vitrification offers substantial advantages over traditional methods of cryopreservation. This eliminates the need for expensive programmable freezers and requires considerably less time, approximately 10 min compared to 1 h for slow cooling. Furthermore, vitrification demonstrates superior post-thaw sperm quality compared to conventional slow freezing methods (30). However, both of these techniques have different methodologies and disadvantages that can cause cell defects during the cryo process, as shown in the Figure 2.

Recent innovative techniques and their impact on sperm

Oocyte and embryo vitrification have become standard cryopreservation techniques due to their significant benefits in assisted reproductive technologies. However, techniques of vitrification for spermatozoa were hard to come by because of their peculiarities (31). For instance, spermatozoa are osmotically delicate, and using highly concentrated, permeable CPAs is hazardous and may occasionally lead to genetic malfunction in the sperms. Consequently, earlier attempts at vitrification were made either without CPAs or with trace levels of CPAs. By using modest volumes of CPAs, the surface area to volume ratio of the preserved sample was increased and this facilitated a quick temperature shift and rapid cooling rates. The post-thaw sperm characterization outperformed the slow freezing method when the vitrification solution had a low concentration of CPA medium containing trehalose (32).

The high CPA levels used in vitrification techniques could be toxic for cells (33). Current recommendations suggests combining multiple CPAs to mitigate such potential risks. Employing a mixture of CPAs reduces individual component concentrations below their cytotoxic thresholds, thereby minimizing cellular stress. Furthermore, this approach reduces the exposure duration of oocytes and embryos to the vitrification solution, enhancing their overall viability and post-thaw survival (34). Some frequently used CPA solutions are constituted of permeating agents such as ethylene glycol, glycerol or dimethyl sulfoxide.

Innovative techniques for assessing sperm function following cryopreservation include evaluations of sperm vitality, motility, and morphology. For instance, eosin-nigrosin staining is commonly employed to assess sperm viability, with analyses typically conducted on a minimum of 200 spermatozoa per sample (35). The Computer-Assisted Sperm Analysis (CASA) system has been used to study sperm motility and morphology, allowing for the

determination of several motion parameters, including progressive, nonprogressive, immotilities, straight, and curved velocity lines. Sperm morphology is also assessed using the WHO criteria, where at least 200 sperm cells are evaluated for head, mid-piece and tail abnormalities, in addition to excessive cytoplasmic droplets (36).

Another method that has been employed is lyophilization. Although lyophilized sperm is a dead structure, sperm chromatin structure is maintained, and the DNA integrity is not altered (37). Technology is now available that permits fertilization using dead sperm and oocyte (38). More human studies are necessary to prove the effectiveness of this technique. The ability to preserve the samples at 4 °C is appealing and effective as it allows for short-term storage while maintaining sperm viability and function.

OOCYTE CRYOPRESERVATION

The first pregnancy resulting from an already cryopreserved oocyte reported in 1986. With advanced technology, using slow freezing to ultra rapid vitrification methods, there has been a considerable increase in oocyte survival and clinical pregnancy rate (39). Human oocyte cryopreservation is dependent on morphological and biophysical parameters that might affect oocyte viability on post-thaw. The Human Fertilisation and Embryology Authority (HFEA) authorized thawed oocytes for infertility treatment in the UK from the year 2000 (40). The rules of HFEA allow the freezing of gametes for a standard 10-year storage and maybe for a longer period under some circumstances.

Challenges and advances in vitrification protocols for oocytes

Mammalian oocytes have long been considered resilient cells with a low surface area to volume ratio and a high susceptibility to intracellular ice formation (41). Some of these early studies raised issues about how the cell membrane permeability qualities of female oocytes evaluated with other biophysical

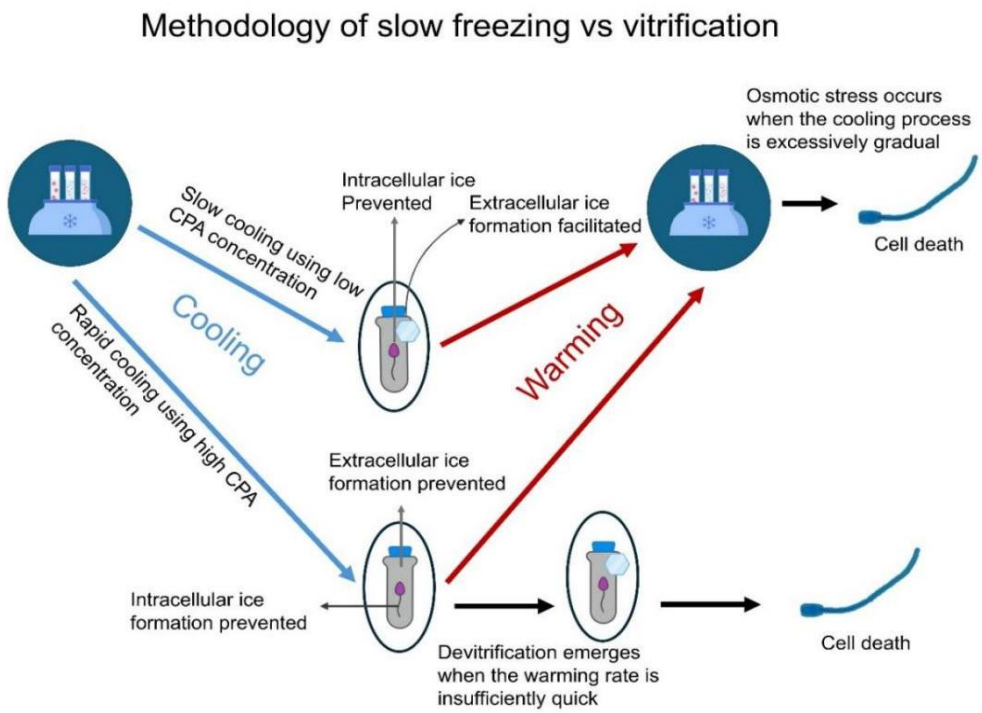

Figure 2. Slow freezing and vitrification differ in their cooling and warming processes. In slow freezing, low concentrations of cryoprotective agents (CPAs) are used, preventing intracellular ice formation but facilitating extracellular ice formation, which can lead to osmotic stress and cell death if the cooling rate is excessively slow. In vitrification, rapid cooling with high CPA concentrations prevents both intracellular and extracellular ice formation, minimizing ice crystal damage. However, inadequate thawing rates results in devitrification, leading to cell death.

features. Research has shown that cryopreservation is undesirable for the preservation of microtubules and the microfilament layers in ovulated cells in mammals (42). The most critical biophysical factor concerned oocyte survival is intracellular ice formation, which commonly penetrates the membrane leading to cell rupture. Since oocytes are large cells capable of holding a significant volume of water, it requires long duration to accomplish an adequate level of dehydration.

Oocyte cryopreservation is vital to every woman with a medical condition, for example endometriosis preparing for surgery (43), low ovarian reserve and those undergoing a sex change operation. Moreover, endo-fertility oocyte cryopreservation is increasingly used for fertility preservation in women with a severe form of endometriosis (44). Despite the growing need for oocyte cryopreservation, research in this area has been comparatively neglected until quite recently.

Progress may have been hindered by the oocyte's inherent susceptibility to physical and chemical stress, which led to poor survival, fertilization, and pregnancy outcomes (45).

However, advancements in this field are accelerating and ethical concerns regarding the storage of excess embryos have been addressed. As a result, oocyte cryopreservation is developing into a reliable and effective method for preserving fertility (46). Mature oocytes now survive better with current cryopreservation technologies. For example, changes in the ratio and composition of CPAs in the slow freezing procedure have improved the rates of subsequent M-II oocyte (Metaphase 2) survival (47).

A comparison of vitrification with slow-freezing cycle data for clinical outcomes was found for a single centre study from HOPE Registry. When using donor oocytes, clinical pregnancy rates and live birth rates were better for vitrification than slow freezing (48). Significant variations in clinical pregnancy rates and live birth yields from vitrified oocytes in autologous versus heterologous cycles were discovered by the same study. There was no difference in clinical outcomes between fresh and donor vitrified oocytes in a randomized controlled trial (RCT): for women treated with vitrified or fresh oocytes, the implantation rate (40% and 41%, respectively), correction rate per cycle (50% in both groups), and correction rate per transfer (55% in both groups) were typically comparable (49).

Effects on oocyte viability and developmental potential

New studies reveal that vitrification significantly enhances oocyte cryopreservation outcomes, leading to higher survival and pregnancy rates. Based on the present information, vitrification is considered the gold standard for oocyte freezing. Advancements in technology have greatly improved the final results and increased its application in clinical practice (50). At the moment, it could be considered a viable option for infertile patients as well as for women with certain medical conditions or no desire to become pregnant for nonsurgical reasons, and its benefits are apparent already.

In a retrospective cohort study the results of women having autologous embryo transfer utilizing frozen vs fresh oocytes were investigated. It was observed that there was no significant difference in the fertilization rates of vitrified-warmed oocytes and fresh oocytes, which were 69.5% and 71.7%, respectively (51). Research has been conducted for retrospective observational study to evaluate Intra-Cytoplasmic Sperm Injection (ICSI) fertilization rates using fresh and frozen donor oocytes. This case-control study utilized endogenous telemedicine to analyze and compare outcomes. Oocyte survival was 96.4%, and neither the clinical pregnancy rate (60.5% and 63.6%) nor the rate of fertilization (83.4% and 86.2%) differed statistically significantly between fresh and vitrified oocytes (52). Comparable rates of fertilization and pregnancy between fresh and frozen donor oocyte cycles were investigated in another retrospective observational research that was carried out that same year (53). However, a study using Society for ART (SART) data from 2013 to 2015 showed that fresh oocytes had a far higher live birth rate (51.1% vs. 39.7%) than vitrified oocytes (54).

Artificial intelligence has been suggested as a potential predictor of the cryopreservation of oocytes. For example, the rate of live birth and fertilization from frozen oocytes has been predicted using an innovative non-surgical technique that incorporates artificial intelligence (55). The process of oocyte grading involves comparing images of recovered mature oocytes with a database of previously cryopreserved eggs that have the potential to grow into blastocysts. In consequence, the probability of a selected oocyte developing into a blastocyst after fertilization, resulting live births, can be

anticipated. Hybrid in vitro fertilization is another new method that can eliminate different outcomes of oocyte cryopreservation in reproductive centers around the world (56).

EMBRYO CRYOPRESERVATION

The preservation of oocyte and embryo germplasm serves as a vital strategy for conserving female genetic material. However, ovarian tissue or entire ovary may be cryopreserved for transplant followed by oocyte retrieval or spontaneous conception. Various methods can help to gather germplasm during certain phases of maturation, including (i) natural, (ii) after spontaneous ovulation, (iii) after stimulated ovulation or by ovum pick up method either by laparoscopic, transvaginal or transrectal (57), (iv) during the natural oestrus cycle in order to induce ovulation when superovulation is desired, (v) after ovariectomy where the ovaries are removed due to diseases, for birth control or postmortem (58), (vi) during early pregnancy (following mating through natural or artificial means, such as AI) at different stages prior to implantation. No matter if studied within groups of primordial and preantral or antral follicles, each degree of feature representing each individual needs and sensitivity can be featured in the collected oocytes. In order to encourage germplasm preservation, it is essential to improve other associated assisted reproductive technologies—IVM, IVF, IVC, and ET.

Conventional slow freezing vs. vitrification techniques

Although the conventional slow freezing method is most frequently employed, variations in pregnancy efficiency are known to exist.

Nevertheless, embryos cryopreserved at the 2PN stage using the vitrification-rapid freezing technique are more ideal in terms of survival rate, which is 81-93% (59). In contrast, the efficiency of conventional slow freezing cryopreservation is around 80% for cleavage stage embryos (60). There are few reports on human cleavage-stage embryo vitrification. Recently, vitrification using cleavage-stage embryos has increased pregnancy rates to 49%.

After gradual freezing or vitrification, embryos may be centrifuged. Four cell-stage human embryos survived better following vitrification than slow freezing (98 % vs. 91 %). Cryopreservation through gradual freezing (47%–53%) or vitrification (51%), does not substantially affect pregnancy rates. Progressive freezing and vitrification were compared in a study of 3-day embryo cryopreservation, showing that vitrified embryos had far greater success rates—which is 95% survival, 35% pregnancy, and 14% implantation—than slow-frozen embryos, which showed 60% survival, 17% pregnancy, and 4% implantation. Similarly, a randomized controlled study also investigated the outcomes of slow freezing for human Day 3 embryos following vitrification, specifically examining intrauterine survival, embryonic energy metabolism, and blastocyst growth (61).

Controversy also arises on the appropriate stage of human embryos development at which they are best cryopreserved. The disadvantage of zygote-stage embryos is that there is no concern as to the ability of the embryo to develop (62). On the other hand, one of the features of cleavage-stage embryos is the fact that sometimes some of blastomeres are damaged and after the thawing those damaged, surviving blastomeres are present just alongside the intact ones. The implantation potential of such embryos is substantially lower than that of entirely viable embryos (63). In contrast to early-stage embryos, working with blastocysts may be more advantageous, as the loss of some smaller cells during cryopreservation is likely to have a lesser impact on the embryo's subsequent development. Since blastocysts are better adapted to the uterine environment, use of an optimized culture system can effectively enhance pregnancy rates in assisted reproductive technology (ART). (64). Since only a small percentage of blastocysts typically survive the transfer stage and are thus eligible for cryopreservation, a standard that would ensure an appropriate and effective procedure regarding the cryopreservation of blastocysts is crucial.

Vitrification technology is more effective for cryopreservation compared to slow freezing and thawing. Since the first pregnancy with vitrified human blastocyst, there has been tremendous interest in vitrification owing to considerably better survival rates and improved pregnancy success. The widespread implementation of vitrification process results in a significant increase in pregnancies, achieved through vitrified blastocysts. (65). Okutsu-Horage showed that the congenital anatomic abnormality rate was the same (1.4%) for infants born from vitrified and fresh blastocyst transfer

(66). Consequently, Liebermann and co-workers found no issues with babies born following vitrification of embryos or gradual freezing (67).

NOVEL CRYOPROTECTANTS

CPAs are important in preserving biological cells within a subzero environment that act to facilitate ice crystal formation. The cryoprotective agent (CPA) used in cryopreservation should remain within an established clinical threshold for biocompatibility, high efficacy and ease of removal from the cryopreserved cells due to balance between lower viscosity (good permeability) and high viscosity (68). Unfortunately, uniting these properties into a single CPA remains difficult. However, biocompatible amino acids, such as β-alanine, γ-aminobutyric acid, and ε-aminocaproic acid, exhibit key characteristics of an effective CPA. The results suggest that they may protect cells from both extracellular and intracellular environments, by reducing the extent of ice formation and osmotic stress, which would be expected to result in a high cryopreservation efficiency for anuclear as well as nucleated mammalian somatic cells (69). Importantly, the efflux of neutral amino acids from cryopreserved cells in a one-stage protocol does not appear to have a significant adverse effect, as cells are able to regulate osmotic balance through crypto-osmotic control mechanisms, which involve passive and active regulation of intracellular solutes to mitigate osmotic stress and maintain cellular homeostasis during cryopreservation and thawing. Nonetheless, these neutral amino acids exhibit potential as improved CPAs compared to conventional osmotic protectants such as DMSO and glycerol, offering enhanced biocompatibility and effective cryoprotection while minimizing cytotoxicity. These findings provide a new point of view in the design of novel CPAs that could be useful in advancing cryopreservation technology.

Development of new cryoprotectants: properties and mechanisms

Living cells, as the material of cell-based medical applications today or in future are essential. For the best results, the collected cells must be healthy enough to keep their viability and function after cryopreservation in the presence of CPAs to prevent ice and osmotic damage from occurring in cells during freezing process (70). Dimethyl sulfoxide (DMSO) and glycerol, two of the most effective cryoprotective agents (CPAs), are well known for their excellent cryoprotective properties. (Table 1). Nevertheless, important limitations largely restrict their clinical use. For instance, they can deliver intrinsic toxicity that will result in damage to cells and/or clinical safety issues. Since DMSO affects enzyme activity, it can combine with glycerol to cause serious intravascular haemolysis.

Many methods have been proposed to reduce the toxic effect of these CPAs, in order to overcome limitations of conventional vitrification. Several studies have, for example, developed toxicity cost functions to support the CPA addition and removal operations in a way that minimizes toxicities (71). Additionally, DMSO and glycerol have poor membrane permeation properties which require multiple washing steps to purge them from cryopreserved cells. The technique, however, is time-consuming, and may lead to a substantial loss of cells during the washing steps (~10%), slowing down the timely delivery of urgently needed life-saving cell products (72). Accordingly, CPAs with properties such as biocompatibility, high transfection efficiency and rapid release from the cell have tremendous value in their design.

To this end, a number of CPAs have been developed in the last few decades to substitute or reduce DMSO/glycerol content altogether: antifreeze proteins AF(G)P, with their ability to suppress ice recrystallization has led them to be particularly well studied. Nevertheless, since AF(G)Ps are alien, they elicit an immune response. Still, it is challenging to biosynthesize AF(G)Ps in large quantities for a variety of uses or to get them from natural sources (73). Many plants accumulate osmoprotectants (e.g., betaine, proline) to increase the freezing tolerance for cold stress and some researchers have reported recently that these protective compounds are very promising CPAs. For example, they help regulate intra- and extracellular osmotic pressure, stabilize protein function after freezing (74), and have been linked to neutral amino acids (nAAs). The observed connections between osmoprotectants, AF(G)Ps, and other polar solutes such as cations, suggests that a particularly intriguing aspect of contemporary study is its broader

"web-of-life" perspective on molecular adaptations. This perspective highlights the interconnected roles of these three classes of solutes, all fundamentally linked to cellular survival. Furthermore, emerging discussions suggest a potential relationship between these solutes and neutral amino acids, further expanding the understanding of cryoprotective mechanisms. Structure analysis of AF(G)Ps shows it is enriched with neutral amino acid and has a number of charged nAAs. About 70% of type I AFPs have neutral alanine residues, highlighting their function as osmoprotectants. Many of the osmoprotectants are neutral amino acids or their derivatives (75). Several nAA transporters on the cell membrane facilitate efficient uptake and release of various molecules, playing a crucial role in mitigating osmotic damage to cells. The carboxy and amino group of nAAs can form hydrogen bonds with water molecules. This interaction may disrupt the connections between water molecules and inhibit ice formation. As a result, during the cryopreservation process, nAAs may lessen the osmotic and mechanical damage that ice causes to cells (76). As of now, detailed research on the role of nAAs in cell cryopreservation, particularly their ability to inhibit ice formation and reduce osmotic stress, remains insufficient. A summary and comparative overview of CPAs' properties, applications, and limitations is presented in Table 1 (77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89).

Comparative efficacy and toxicity profiles and its applicability for cryosurvival

CPA toxicity is recognized as the primary challenge in achieving successful organ cryopreservation through vitrification. Gaining a deeper understanding of how CPA toxicity works and finding ways to mitigate it could be crucial for effective organ preservation (90). The toxicity of CPA differs among cells, as reported by several cryopreservation studies. However, all cells share similar biological components and macromolecules. Investigating why toxicities differ in various biological contexts can enhance our understanding of CPA toxicity mechanisms. For instance, embryos from one species exhibit substantial differences in CPA toxicities and so need comparative studies to elucidate the underlying mechanisms and enhance understanding of toxicity pathways (91).

A significant portion of the discrepancies in CPA toxicity studies arises from variations in experimental conditions, including CPA concentration, exposure duration, carrier solutions. CPAs can be harmful if they compromise cell membranes, impair enzyme activity, affect mitochondrial function, or disrupt DNA, proteins, or other macromolecules. Furthermore, certain effects linked to CPA toxicity may result from other types of damage, such as osmotic shock, oxidative stress, or chilling injury (92). Two forms of toxicity exist: non-specific toxicity, which is brought on by being a CPA, and specific toxicity, which is particular to a single CPA. It is believed that CPAs prevent ice from forming by breaking the hydrogen bonds that hold water molecules together (93). Furthermore, certain effects linked to CPA toxicity may result from other types of damage, such as osmotic shock, oxidative stress, or chilling injury compounds, which might result in toxicity that is not specific. Further research is essential to explore the multifunctional roles of penetrating CPAs, including ethylene glycol, propylene glycol, dimethyl sulfoxide, glycerol, formamide, methanol, and butanediol.

Table 1. Comparative overview of cryoprotective agents (CPAs): properties, applications, and limitations.

Cryoprotect-ant / Type	Features / Applications	Merit / Demerit	Ref
Dimethyl sulfoxide (DMSO) / permeable	Features: Commonly used; penetrates cell membranes; reduces ice formation. Applications: Stem cells, embryos, sperm preservation.	Merit: High efficacy in preventing ice formation. Demerit: Cytotoxic at high concentrations.	(77)
Glycerol / permeable	Features: Reduces intracellular ice formation; widely used in sperm cryopreservation. Applications: Sperm banking, various cell types.	Merit: Low toxicity compared to other CPAs. Demerit: Limited effectiveness in some cell types.	(78)
Ethylene glycol / permeable	Features: Effective in reducing ice crystal formation; used for oocytes and embryos. Applications: Oocyte and embryo preservation.	Merit: Good vitrification properties. Demerit: Potential for cellular toxicity at high levels.	(79)
Butane-2,3-diol / permeable	Features: Comparable to DMSO; better physical characteristics for vitrification. Applications: Tissue and cell preservation.	Merit: Lower toxicity and effective at low concentrations. Demerit: Limited research on long-term effects.	(80)
Polyvinyl alcohol (PVA) / non-permeable	Features: Stabilizes cells during freezing; reduces ice crystal growth. Applications: Stem cells, various tissues	Merit: High biocompatibility and effectiveness in cryoprotection. Demerit: May require combination with permeable CPAs.	(81)
Antifreeze proteins (AFPs) / natural	Features: Inhibits ice recrystallization; enhances cell viability post-thawing. Applications: Sperm, embryos, various cell types.	Merit: Significant improvement in post-cryopreservation viability. Demerit: Limited availability and high cost of extraction	(82)
Poly-ampholytes / synthetic	Features: Unique structure inhibits ice nucleation; effective at low concentrations. Applications: Stem cells, tissue engineering.	Merit: Excellent ice protection properties. Demerit: Complexity in synthesis and application.	(83)
Hydroxy-ethyl starch (HES) / non-permeable	Features: Stabilizes cells during freezing; low toxicity; enhances cryopreservation outcomes. Applications: Blood products, tissues	Merit: Biocompatible and effective for various applications. Demerit: Limited penetration into cells may reduce efficacy.	(84)
Alginate / non-permeable	Features: Provides mechanical support; can dehydrate cells before freezing. Applications: Stem cells, erythrocytes, tissue engineering.	Merit: High survival rates without permeable protectants. Demerit: Requires careful handling to avoid gel formation issues.	(85)
1,2-Propanediol / permeable	Features: High glass-forming tendency; stable amorphous state during cooling/warming. Applications: Vitrification of various cell types.	Merit: Excellent for maintaining cellular integrity during freezing. Demerit: Less effective than DMSO in some applications.	(86)
Betaine / zwitterionic	Features: Natural compound that enhances cell survival during cryopreservation processes. Applications: Whole blood cryopreservation, gametes	Merit: Low toxicity and biocompatibility with high efficacy. Demerit: Less studied compared to traditional CPAs.	(87)
1,3-Cyclo-hexanediol / synthetic	Features: Effective at controlling ice growth; tested in various concentrations for efficacy. Applications: Various cell types including gametes	Merit: Enhanced cryoprotective properties compared to traditional agents. Demerit: Limited research on long-term effects on viability.	(88)
Nano-materials (e.g., Graphene) / synthetic	Features: Enhanced cell survival rates observed with innovative materials for cryoprotection. Applications: Various biological applications	Merit: Potential for improved efficacy over traditional CPAs. Demerit: Still experimental with limited clinical application data.	(89)

IMPACT ON FERTILITY PRESERVATION

Nearly 75% of women between the ages of 18 and 45 who have received a recent cancer diagnosis are eager to start a family, according to statistics (94). However, factors including reduced ovarian reserve, early menopause caused by gonadotoxic drugs, or surgical removal of reproductive organs can all lead to reproductive issues. Alkylating drugs including cyclophosphamide, ifosfamide, nitrosoureas, and procarbazine are extremely harmful to the ovaries and increase the chance of infertility, with ovarian failure rates ranging from 15% to 50% (95). When myeloablative stem cell transplantation is combined with whole body irradiation, the likelihood of permanent amenorrhea may exceed 80%. Women of reproductive age have stated that the potential loss of fertility can be nearly as distressing as coping with disease, if not more so, and that the prospect of having children after cancer may be a powerful motivator for recovery. A survey of cancer patients revealed that over half of them considered having a child to be the "most important" aspect of their lives, and 62% of them were "most concerned" about how cancer treatment could affect their ability to conceive. Concerns about infertility brought on by therapy might have a big impact on important treatment decisions. Concerns about infertility affected treatment decisions for 29% of 657 women of young women with breast cancer in a large online study (96). Furthermore, a tiny number (1%–11% of 620 women of women under 40 who had just received an early-stage breast cancer diagnosis (in another prospective multicenter research program) thought about refusing, reducing, or altering their chemotherapy or endocrine treatment in order to protect their long-term fertility.

Individuals looking to protect their reproductive options may seek advice from a reproductive medicine specialist, often referred to as fertility preservation counselling (FPC). These consultations can cover various topics, including fertility statistics influenced by demographic factors and cancer treatments, the potential impact of future pregnancies on cancer recurrence risk, and the success rates of different fertility-preserving techniques, include preimplantation genetic testing and donor egg and surrogate alternatives (97). Following that, some women might decide to have fertility preservation, usually using the gold standard of embryo cryopreservation. Oocyte cryopreservation is an additional, albeit less accessible, option that may be attractive to women who are single or just starting a relationship.

During embryo cryopreservation, the rates of pregnancy for infertile women without cancer differ with age, exceeding 42% for women under 35, 26%-40% for those aged 35-42 years old, and less than 20% for women over 42 years (98). When carried out by qualified specialists, similar patterns have been observed for oocyte cryopreservation in cancer-free infertile women. Pregnancy rates among cancer patients after oocyte or embryo cryopreservation are, however, little documented. A new study suggests that the cumulative live birth rates per embryo transfer may be comparable for cancer patients and age-matched infertile controls (99). Additionally, there are experimental options for fertility preservation, such as ovarian tissue cryopreservation and temporarily relocating ovaries away from radiation exposure. Finally, goserelin appears to help preserve ovarian function and improve future reproductive prospects when administered after adjuvant treatment (100).

CLINICAL OUTCOMES OF ADVANCED CRYOPRESERVATION METHODS

The therapeutic environment in fertility care is evolving due to the demand for ART and the development of cryopreservation procedures. The need to maintain fertility, as well as evolving societal trends, are driving the increasing demand for assisted reproductive technologies. These advancements, along with a deeper understanding of factors influencing ART outcomes, continue to improve success rates. However, newly identified factors, such as sperm DNA fragmentation, endometrial and vaginal microbiota, may impact treatment efficacy and require further investigation (101). Transferring all the high-quality embryos produced in a new cycle to later natural or artificially prepared cycles after they have been cryopreserved is known as a "freeze-all" strategy. The method, commonly referred to as "freeze-only," was initially documented in the literature over two decades ago. Early studies identified its use in protocols that delay

Table 2. Advances in cryopreservation techniques: features, outcomes, and applications.

Cryopreservation methods	Features	Outcomes	Application	Ref
Vitrification of Oocytes	Rapid freezing reduces ice crystal formation; enhances fertilization rates.	Post-thaw survival rates improved to 75% with 1,2-propanediol as cryoprotectant.	Oocyte preservation	(109)
Cryopreservation of sperm with glycerol	Glycerol penetrates cells, protecting against ice damage.	Viability rates post-thaw around 60-70%; effective for long-term storage.	Sperm banking	(110)
Use of antifreeze proteins (AFPs)	Inhibits ice recrystallization; biocompatible.	Enhanced viability in sperm and embryos; improved post-thaw outcomes.	Sperm, embryos	(111)
Polyampholytes with DMSO	Macromolecular cryoprotectants enhance cell integrity during freezing.	Reduced membrane damage; higher recovery rates compared to traditional methods.	Sperm, oocytes	(112)
Cryopreservation of embryos via vitrification	Cryopreservation of embryos via vitrification	Live birth rates from cryopreserved embryos increased significantly post-thawing.	Embryo storage	(113)
Rapid cooling techniques for sperm	Fast cooling minimizes ice formation; preserves cellular integrity.	Improved motility rates post-thaw; effective for ART procedures.	Assisted reproductive technology (ART)	(114)
Cryopreservation of oocytes with ethylene glycol	Ethylene glycol used in combination with other agents for enhanced outcomes.	Fertilization rates improved to 80% after thawing; reduced polyploidy rates.	Oocyte preservation	(115)
Nanomaterials in cryoprotection	Innovative materials enhance traditional cryoprotectant efficacy.	Increased cell survival rates observed with graphene-based cryoprotectants.	Various biological materials	(116)
Chloro trifluoro methane (CCIF3) method	Ideal immersion times enhance tissue preservation outcomes.	Effective tissue processing with minimal damage noted post-cryopreservation.	Tissue engineering	(117)
Cryopreservation of bioartificial liver constructs	Alginate encapsulation improves survival rates during cryopreservation.	Retained hepatocyte function post-thawing; effective for liver failure management.	Liver transplantation	(118)
Combination cryoprotectants for embryos	Utilizes a combination of cryoprotectants to optimize embryo viability post-thaw.	High survival rates (50-85%) reported in children born from frozen embryos without increased defects.	Embryo storage and transfer	(119)
Electrostatic spray method for sperm	Advanced delivery technique for even distribution of cryoprotectants.	Improved sperm motility and viability post-thaw compared to traditional methods.	Sperm banking	(120)
Cryopreservation of neural stem cells (NSCs)	Enhanced protocols improve viability and functionality after thawing.	NSC spheres achieved a survival rate of 82.9% post-thawing using optimized protocols.	Stem cell therapies	(121)
Cryopreservation of testicular tissue	Innovative techniques allow preservation of male fertility options.	Successful recovery and maturation of spermatogonial stem cells after thawing.	Male fertility preservation	(122)
Use of hydrogel-based encapsulation techniques	Hydrogel matrices provide protection during freezing and thawing processes.	Enhanced post-thaw viability and functionality in various cell types, including gametes.	Various biological applications	(123)

implantation to reduce the risk of ovarian hyperstimulation syndrome (OHSS). Recently,

the "freeze-all" strategy has been increasingly adopted in conjunction with preimplantation genetic testing (PGT) to optimize embryo selection and to improve clinical outcomes (102). The entire procedure might be better described by the more recent term elective frozen embryo transfer (eFET). Embryo and oocyte cryopreservation procedures and strategies are expected to improve IVF outcomes (103).

Future cryopreservation applications in ART can be categorized into elective and non-elective. Elective cryopreservation includes fertility preservation for social or medical reasons, while non-elective cryopreservation is for medical conditions or treatment protocols that may compromise reproductive potential (104). The former depends on the patient's decision, whilst the latter is brought about by medical indications and a lack of additional reproductive options. In the past, women with medical indications who had no other options for reproduction were given consideration for cryopreservation of embryos or oocytes. Oocyte donation and social freezing of oocytes are the most popular forms of voluntary cryopreservation, which has expanded with the growing use of cryopreservation (105). In order to increase the chances of future IVF success in cases of multiple implantation failure, some patients may decide to have clinical oocyte freezing, which involves retrieving more oocytes during subsequent rounds of ovarian stimulation.

Another topic that is being studied more and more is transgender fertility preservation. Men with damaged DNA are more likely passing their multifactorial disorders to their offspring. In females who prefer not to cryopreserve embryos, oocyte cryopreservation provides a workable substitute. It also tends to avoid the ethical issues or legal restrictions that may be associated with the storage and cryopreservation of embryos, as well as any potential disputes that might arise if the couple later splits up. However, there is evidence from several observational studies that utilizing frozen-thawed embryos leads to higher implantation and pregnancy rates than using embryos generated from frozen oocytes, and the science of embryo cryopreservation is well-established (106).

The primary medical indication for elective embryo cryopreservation is PGT-A (preimplantation genetic testing for aneuploidy), which provides enough time to identify an appropriate euploid embryo for transfer. In a facility where eFET (elective frozen embryo transfer) is available, it may be the first option for a number of reasons besides medical ones (107). Even though the published data reviewed indicates that eFET performs better than fresh embryo transfer in a few specific populations, including high responders, women with polycystic ovary syndrome (PCOS), and in PGT-A cycle settings alone, where FET does not seem to be less effective than fresh embryo transfer in normal responders, cryopreservation will never be a reason for concern (108).

Researchers are continually looking to improve cryopreservation outcomes by designing new CPAs, the use of new materials and methods, as summarized in Table 2 (109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123). For example, one focus has been on natural CPAs as substitutes for synthetic CPAs.

CONCLUSION

Cryopreservation relies on cryoprotective agents since they shield cells from damage during freezing and thawing. Even though synthetic CPAs like DMSO and glycerol are using widely, natural cryoprotectants have gained interest due to their potential biocompatibility and reduced toxicity. However, their effectiveness and mechanism of action in cryopreservation remain unexplored.

Further research is needed to identify more cost-effective cryoprotectants and to better understand their impact on cell viability. Additionally, recent studies have highlighted the potential epigenetic changes induced by CPAs, emphasizing the need for continued investigation into their long-term effects. Even at low doses, exposure to DMSO has been shown to change the epigenetic profile of embryonic stem cells. In some situations, this alteration may also have positive impacts on gametes by influencing cellular reprogramming or resilience. However, the specific processes behind these impacts are yet unclear. Conducting extensive studies on CPA toxicity is essential since new cryoprotectants and improved vitrification methods keep emerging. Clarifying the degree of epigenetic changes, their possible reversibility, and any long-term effects on cellular growth, function, and general

viability should be addressed in cryopreservation.

This review compiles current findings on cryoprotectants and their significance in cryopreservation, highlighting the need for further research in this field. Although there has been significant progress in developing safer and more efficient CPAs, challenges remain in understanding their long-term biological effects, including potential epigenetic changes.

REFERENCES

1. Chandi A & Jain N (2021) *Biol. Reprod.* **105(4)**, 808-821. https://doi.org/10.1093/biolre/ioab122
2. Meneghel J, Kilbride P & Morris GJ (2020) *Front. Med.* **7,** 592242. https://doi.org/10.3389/fmed.2020.592242
3. Marcantonini G, Bartolini D, Zatini L, Costa S, Passerini M, Rende M, Luca G, Basta G, Murdolo G, Calafiore R & Galli F (2022) *Molecules* **27(10)**, 3254. https://doi.org/10.3390/molecules27103254
4. McClam M & Xiao S (2022) *Biol. Reprod.* **106(2),** 328-337. https://doi.org/10.1093/biolre/ioac008
5. Bosch E, De Vos M & Humaidan P (2020) *Front. Endocrinol.* **11**, 67. https://doi.org/10.3389/fendo.2020.00067
6. Teo UL, Kakkar P & El-Toukhy T (2022) *Obstet. Gynaecol.* **42(3),** 370-8. https://doi.org/10.1080/01443615.2021.1904220
7. Nagy ZP, Shapiro D & Chang CC (2020) F&S 113(2), 241-247. https://doi.org/10.3389/fendo.2023.1193826
8. Pathy M R, Vickram AS, Sridharan TB, Parameswari R, Archana K, Nithya S, Mishika A (2017) *CryoLetters* **38(6)**, 434-444.
9. Mochida K (2020) *J. Reprod. Dev* **66(4)**, 299-306. https://doi.org/10.1262/jrd.2020-033
10. Contreras MJ, Arias ME, Fuentes F, Muñoz E, Bernecic N, Fair S & Felmer R (2023) *J Equine Vet Sci* **126,** 104499.
11. Estudillo E, Jiménez A, Bustamante-Nieves PE, Palacios-Reyes C, Velasco I & López-Ornelas A (2021) *Int. J. Mol. Sci.* **22(19)**, 10864. https://doi.org/10.3390/ijms221910864
12. Ozturk S (2020) Mol. Reprod. Dev. 87(10), 1021-36. Pomeroy KO, Comizzoli P, Rushing JS, Lersten IL & Nel-Themaat L (2022) *Fertil Steril.* **117(3)**, 469-476.
13. Boafo GF, Magar KT, Ekpo MD, Qian W, Tan S & Chen C (2022) *Int. J. Mol. Sci.* **23**, 12487. https://doi.org/10.3390/ijms232012487
14. Weng L & Beauchesne PR (2020) *Cryobiology* **94**, 9-17. https://doi.org/10.1016/j.cryobiol.2020.03.012
15. Lomba L, García CB, Benito L, Sangüesa E, Santander S & Zuriaga E (2023) *ACS* **10**, 178-190. https://doi.org/10.1021/acsbiomaterials.3c00859
16. Eskandari A, Leow TC, Rahman MB & Oslan SN (2020) *Biomolecules* **10,** 1649. https://doi.org/10.3390/biom10121649
17. Han H, Zhan T, Guo N, Cui M & Xu Y (2024) *Biotechnol. J.* **19(2)**, 2300543. https://doi.org/10.1002/biot.202300543
18. Rockinger U, Funk M & Winter G (2021) *Journal of Pharmaceutical Sciences* **110(8)**, 2873-2893. https://doi.org/10.1016/j.xphs.2021.04.018
19. Jungare KA, Radha R & Sreekanth D (2022) *Mater. Today Proc.* **51**, 1637-1641. https://doi.org/10.1016/j.matpr.2021.11.203
20. Borate GM & Meshram A (2022) *Cureus* **14(11)**, e31402
21. Tao Y, Sanger E, Saewu A & Leveille MC (2020) *Reprod. Biol. Endocrinol.* **18(1)**, 17. https://doi.org/10.1186/s12958-020-00580-5
22. Chang T & Zhao G (2021) *Adv. Sci.* **8(6)**, 2002425.
23. Bai B, Xue C, Wen Y, Lim J, Le Z, Shou Y, Shin S & Tay A (2023) *Adv. Funct. Mater.* **33(40),** 2303373. https://doi.org/10.1002/adfm.202303373
24. Huang C, Tang YL, Hu JL, Zhou WJ, Huang ZH, Luo XF, Li Z & Zhu WB (2022) *Asian J. Androl.* **24(6)**, 563-569.
25. Upadhyay VR, Ramesh V, Dewry RK, Kumar G, Raval K & Patoliya P (2021) *Andrologia* **53(8)**, e14154. https://doi.org/10.1111/and.14154
26. Ezzati M, Shanehbandi D, Hamdi K, Rahbar S & Pashaiasl M (2020) *Cell Tissue Bank.* **21(1)**, 1-5. https://doi.org/10.1007/s10561-019-09797-0

27. Cheng Q, Li L, Jiang M, Liu B, Xian Y, Liu S, Liu X, Zhao W & Li F (2022) *Cells* **11(18),** 2845. https://doi.org/10.3390/cells11182845
28. Lin M, Cao H & Li J (2023) *Acta Biomater.* **155,** 35-56. https://doi.org/10.1016/j.actbio.2022.10.056
29. Zamecnik J, Faltus M & Bilavcik A (2021) *Plants* **10(12)**, 2623. https://doi.org/10.3390/plants10122623
30. Cobo A, García-Velasco JA, Remohí J & Pellicer A (2021) *Fertil. Steril.* **115(5),** 1091-1101. https://doi.org/10.1016/j.fertnstert.2021.02.006
31. Gore M, Narvekar A, Bhagwat A, Jain R & Dandekar (2022) *J. Mater. Chem. B.* **10(2)**, 143-169. https://doi.org/10.1039/D1TB01449H
32. Tamburrino L, Traini G, Marcellini A, Vignozzi L, Baldi E & Marchiani S. (2023) *Int. J. Mol. Sci.* **24(5)**, 4656. https://doi.org/10.3390/ijms24054656
33. Liu X, Pan Y, Liu F, He Y, Zhu Q, Liu Z, Zhan X & Tan S (2021) *J. Nanomater.* **2021(1),** 990709. https://doi.org/10.1155/2021/9990709
34. Baskaran S, Finelli R, Agarwal A & Henkel R (2021) *Andrologia* **53(2)**, e13614. https://doi.org/10.1111/and.13614
35. Sudhakaran G, Kesavan D, Kandaswamy K, Guru A, Arockiaraj J (2024) *Reprod Toxicol* **124,** 108531.
36. Popoola PO, Musa A, Faloye AD, Ojediran EO, Olaniran TT, Ayodele ID, Adeyemi KA, Adebayo ET, Ajayi A (2024) *AJBMR* **7(2),** 75-96. https://doi.org/10.52589/AJBMR-RSO6LQ0B
37. Loi P, Anzalone DA, Palazzese L, Dinnyés A, Saragusty J & Czernik M (2021) *Reprod. Fertil. Dev.* **33(2),** 82-90. https://doi.org/10.1071/RD20264
38. Bolton VN, Hayden C, Robinson M, Abdo D & Pericleous-Smith A (2023) *Hum. Fertil.* **26(1)**, 2-16. https://doi.org/10.1080/14647273.2023.2190987
39. Sciorio R (2020) *Gynecol. Endocrinol.* **36(5),** 381-388. https://doi.org/10.1080/09513590.2020.1719402
40. Whaley D, Damyar K, Witek RP, Mendoza A, Alexander M & Lakey JR (2021) *Cell Transplant.* **30**, 0963689721999617. https://doi.org/10.1177/0963689721999617
41. Ribeiro JC, Carrageta DF, Bernardino RL, Alves MG & Oliveira PF (2022) *Animals* **12(3),** 359. https://doi.org/10.3390/ani12030359
42. De Coster T, Velez DA, Van Soom A, Woelders H & Smits K (2020) *Reprod. Fertil. Dev.* **32(5)**, 453-467. https://doi.org/10.1071/RD19229
43. Gazzo I, Moffa F & Ferrero S (2024) *Best Pract Res. Clin. Obstet. Gynaecol.* **16**, 102503. https://doi.org/10.1016/j.bpobgyn.2024.102503
44. Popova E, Kulichenko I & Kim HH (2023) *Biology* **12(4),** 542. https://doi.org/10.3390/biology12040542
45. Go KJ, Romanski PA, Bortoletto P, Patel JC, Srouji SS & Ginsburg ES (2023) Fertil *Steril.* **119(1),** 15-20. https://doi.org/10.1016/j.fertnstert.2022.09.323
46. Jones AS & Shikanov A (2020) *Curr. Breast Cancer Rep.* **12(4),** 351-360. https://doi.org/10.1007/s12609-020-00390-z
47. Walker Z, Lanes A & Ginsburg E (2022) *Reprod. Biol. Endocrinol.* **20(1)**, 10. https://doi.org/10.1186/s12958-021-00884-0
48. Sciorio R, Antonini E & Engl B (2021) Zygote. 29(5), 410-6. https://doi.org/10.1017/S0967199421000204
49. Dujíčková L, Makarevich AV, Olexiková L, Kubovičová E & Strejček F (2021) *Zygote* **29(1)**, 1-11. https://doi.org/10.1017/S0967199420000465
50. Selvakumar SC, Preethi KA, Ross K, Sekar D. (2024) *Placenta* **158**, 38-47.
51. Walker Z, Lanes A & Ginsburg E (2022) Reprod. biol. endocrinol. **20(1),** 10. https://doi.org/10.1186/s12958-021-00884-0
52. Paffoni A, Reschini M, Pisaturo V, Guarneri C, Palini S & Viganò P. (2021) Reproductive Reprod. Biol. Endocrinol. **19,** 1-3. https://doi.org/10.1186/s12958-021-00784-3
53. Kalinderis M, Kalinderi K, Srivastava G & Homburg R (2021) Reprod. Sci. **28(11),** 3061-72. https://doi.org/10.1007/s43032-021-00628-w

54. Pai HD, Baid R, Palshetkar NP, Pai A, Pai RD & Palshetkar R (2021) *J. Hum. Reprod. Sci.* **14(4),** 340-9. https://doi.org/10.4103/jhrs.jhrs_173_21
55. Abdullah KA, Atazhanova T, Chavez-Badiola A & Shivhare SB (2023) *Reprod Sci.* **30(4)**, 1006-16. https://doi.org/10.1007/s43032-022-00941-y
56. Vyrides AA, El Mahdi E & Giannakou K (2022) *Front. Med.* **9**, 982230. https://doi.org/10.3389/fmed.2022.982230
57. Agca C, Klakotskaia D, Stopa EG, Schachtman TR & Agca Y (2020) *JAD* **73(2),** 529-541.
58. Xingzhu D, Qingrui Z, Keren C, Yuxi L, Yunpeng H, Shien Z & Xiangwei F (2021) *Biopreserv Biobank.* **19(3),** 210-218. https://doi.org/10.1089/bio.2020.0074
59. Sekar S, Panchu SE, Gandhi MB, Rajaram V, Nivethaa EA, Sridharan MB, Subbaraya NK (2024) *J. Environ. Chem. Eng* **12(5),** 113738.
60. Shenoy CC, Khan Z, Coddington CC, Stewart EA, Morbeck DE. *Reproductive Sciences* **28(12),** 3473-3479. https://doi.org/10.1007/s43032-021-00758-1
61. Nanassy L, Schoepper B, Schultze-Mosgau A, Depenbusch M, Eggersmann TK, Hiller RA & Griesinger G (2023) *J Assist Reprod Genet.* **40(10)**, 2357-2365. https://doi.org/10.1007/s10815-023-02909-6
62. Li J, Xiong S, Zhao Y, Li C, Han W & Huang G (2021) *Front Endocrinol.* **14,** 12:653310. https://doi.org/10.3389/fendo.2021.653310
63. Ozgur K, Berkkanoglu M, Bulut H, Donmez L, Isikli A & Coetzee K (2021) *J Assist Reprod Genet.* **38**, 1077-1087. https://doi.org/10.1007/s10815-021-02110-7
64. Nagy ZP, Shapiro D & Chang CC (2020) *Fertil. Steril.* **113(2),** 241-247. https://doi.org/10.1016/j.fertnstert.2019.12.009
65. Xu Y, Chan LL, Chen S, Ying B, Zhang T, Liu W, Guo H, Wang J, Xu Z, Zhang X & He X (2021) *Cryobiology* **99**, 131-139. https://doi.org/10.1016/j.cryobiol.2020.11.010
66. Okutsu-Horage Y, Iwahata H, Suzuki-Takahashi Y, Sugishita Y, Takae S & Suzuki N (2022) *Assist Reprod Gen.* **39(8)**, 1769-77.https://doi.org/10.1007/s10815-022-02575-0
67. Sciorio R, Tramontano L, Campos G, Greco PF, Mondrone G, Surbone A, Greco E, Talevi R, Pluchino N & Fleming S (2024) Front. Cell Dev. Biol. 12,1398049. https://doi.org/10.3389/fcell.2024.1398049
68. Dou M, Lu C & Rao W (2022) *Trends Biotechnol.* **40(1)**, 93-106.
69. Jahed KR, Saini AK & Sherif SM (2023) *Front. Plant Sci.* **14**, 1246093. https://doi.org/10.3389/fpls.2023.1246093
70. Figoli A, Marino T, Simone S, Di Nicolò E, Li XM, He T, Tornaghi S, Drioli EJ (2014) *Green Chemistry* **16(9)**, 4034-4059. https://doi.org/10.1039/C4GC00613E
71. Sánchez-Arévalo CM, Vincent-Vela MC, Luján-Facundo MJ & Álvarez-Blanco S (2023) *Process Saf. Environ. Prot.* **177**, 118-137. https://doi.org/10.1016/j.psep.2023.06.073
72. Zhuang H, Hao H, Qiu Y, Gan J & Li T (2023) *Chin. J. Chem.* **41(16),** 2010-24.
73. Omari Alzahrani F (2021) *Planta* **253(1)**, 24. https://doi.org/10.1007/s00425-020-03550-8
74. Cheng CH & Zhuang X (2020) *J. Syst. Evol.* **2020**, 275-313. https://doi.org/10.1007/978-3-030-41929-5_9
75. Ashour M & Omran AM (2022) *Fermentation* **8(9)**, 466. https://doi.org/10.3390/fermentation8090466
76. Awan M, Buriak I, Fleck R, Fuller B, Goltsev A, Kerby J, Lowdell M, Mericka P, Petrenko A, Petrenko Y & Rogulska O (2020) *Regen. Med.* **15(3)**, 1463-1491. https://doi.org/10.2217/rme-2019-0145
77. Saha A, Asaduzzaman M & Bari FY (2022) *Vet. Med. Int.* **2022(1),** 7378379. https://doi.org/10.1155/2022/7378379
78. Olexiková L, Makarevich A, Dujíčková L, Kubovičová E & Chrenek P (2024) *Cryobiology* **116,** 104946. https://doi.org/10.1016/j.cryobiol.2024.104946
79. Petrov K & Petrova P (2021) *Fermentation* **7(4),** 307. https://doi.org/10.3390/fermentation7040307
80. Murray KA & Gibson MI (2022) *Nat. Rev. Chem.* **6(8)**, 579-593. https://doi.org/10.1038/s41570-022-00407-4

81. Eskandari A, Leow TC, Rahman MB & Oslan SN (2024) *Eur. Food Res. Technol.* **250(4),** 1103-21. https://doi.org/10.1007/s00217-023-04449-w
82. Kudaibergenov SE (2021) *Polym. Adv. Technol.* **32(3),** 906-18. https://doi.org/10.1002/pat.5145
83. Wang H, Hu H, Yang H & Li Z (2021) *RSC Adv* **11(6),** 3226-3240. 10.1039/D0RA09663F
84. Sahoo DR & Biswal T (2021) *SN Appl. Sci.* **3(1)**, 30. https://doi.org/10.1007/s42452-020-04096-w
85. Vanderzwalmen P, Ectors F, Panagiotidis Y, Schuff M, Murtinger M & Wirleitner B (2020) *Reprod. Med.* **1(2)**, 142-157. https://doi.org/10.3390/reprodmed1020011
86. Heydarzadeh S, Kheradmand Kia S, Boroomand S & Hedayati M (2022) *Biotechnol. Bioeng.* **119(11),** 2985-3006. https://doi.org/10.1002/bit.28197
87. Solanki PK & Rabin Y (2022) *CryoLetters* **43(1),** 1-9. https://doi.org/10.54680/fr22110110112
88. Chang T & Zhao G (2021) *Adv. Sci.* **8(6)**, 2002425. https://doi.org/10.1021/acsabm.1c00105
89. Chen J, Liu X, Hu Y, Chen X & Tan S (2023) *Front. Vet. Sci.* **10**, 1201794. https://doi.org/10.3389/fvets.2023.1201794
90. Mogas T, García-Martínez T & Martínez-Rodero I (2024) *Reprod Domest Anim* **59**, e14623. https://doi.org/10.1111/rda.14623
91. Yong KW, Laouar L, Elliott JA & Jomha NM (2020) *Cryobiology* **96,** 1-11. https://doi.org/10.1016/j.cryobiol.2020.08.012
92. Landecker H (2024) *BioSocieties* **7**, 1-21. https://doi.org/10.1057/s41292-024-00331-4
93. Lucchini SM, Esteban A, Nigra MA, Palacios AT, Alzate-Granados JP & Borla HF (2021) *Gynecol Oncol.* **161(3)**, 802-809. https://doi.org/10.1016/j.ygyno.2021.04.017
94. Bhardwaj JK, Bikal P & Sachdeva SN (2023) *J Biochem Mol Toxicol* **37(7)**, e23371. https://doi.org/10.1002/jbt.23371
95. El Alaoui-Lasmaili K, Nguyen-Thi PL, Demogeot N, Lighezzolo-Alnot J, Gross MJ, Mansuy L, Chastagner P & Koscinski I (2023) *Cancer Medicine* **12(5),** 6023-6039. https://doi.org/10.1002/cam4.5339
96. Padmavathy B, Ebinezer BS, Amalraj S, Kadaikunnan S, Arumugam M, Karthick V, Karthikeyan K, Prabhu S, Khaled JM, Jose J, Thiruvengadam R (2024) *Mater. Chem. Phy.* **328**, 130030.
97. Fraison E, Huberlant S, Labrune E, Cavalieri M, Montagut M, Brugnon F & Courbiere B (2023) *Hum. Reprod.* **38(3)**, 489-502. https://doi.org/10.1093/humrep/deac249
98. Hussein RS, Khan Z & Zhao Y (2020) *Mayo Clin Proc* 95**,** 770-783. https://doi.org/10.1016/j.mayocp.2019.10.009
99. Cvetanovic AS, Lambertini M, Punie K, Brko GG, Zivkovic ND, Popovic MJ, Kovacevic MM, Popovic LS (2024) *Oncology Research* **32(8),** 1309. https://doi.org/10.32604/or.2024.049743
100. Gao X, Louwers YV, Laven JS & Schoenmakers S (2024) *Int J Mol Sci.* **25(1),** 622. https://doi.org/10.3390/ijms25010622
101. Bourdon M, Maignien C, Pocate-Cheriet K, Bureau GP, Marcellin L, Patrat C, Chapron C & Santulli P (2021) *Reprod. Biomed. Online* **42(3),** 529-545. https://doi.org/10.1016/j.rbmo.2020.11.013
102. Sciorio R & Esteves SC (2020) *Cryobiology* **92,** 9-14. https://doi.org/10.1016/j.cryobiol.2019.11.041
103. Khattak H, Woodman H, Afifi Y, Amorim CA, Fishel S, Gallos I, Coomarasamy A & Topping A (2022) *J Psychosom Obstet Gynaecol* **43(4)**, 502-516. https://doi.org/10.1080/0167482X.2022.2084376
104. Ezeome IV (2022) *Afr J Reprod Health* **26(3)**, 124-135.
105. Jin X, Liu G, Jiao Z, Sun J, Yan M, Lv X, Zhang H & Chen J (2021) *Reprod. Sci.* **28**, 1267-1276. Nhttps://doi.org/10.1007/s43032-020-00323-2
106. Ben Rafael Z (2020) *Hum Reprod* **35(10)**, 2179-2184. https://doi.org/10.1093/humrep/deaa204
107. Kimelman D & Pavone ME (2021) *Best Pract Res Clin Obstet Gynaecol.* **70**, 51-62. https://doi.org/10.1016/j.bpobgyn.2020.07.004
108. Arav A (2022) *Cells* **11(7)**, 1072. https://doi.org/10.3390/cells11071072

109. Ozimic S, Ban-Frangez H & Stimpfel M (2023) *Curr. Issues Mol. Biol.* **45(6),** 4716-4734. https://doi.org/10.3390/cimb45060300
110. Wang X, Wang E & Zhao G (2023) *Cell Regen.* **12(1)**, 28.
111. Anisa I & Irfan y (2024) *Adv Biotech & Micro.* **18(1)**, 555978.
112. Lomba L, García CB, Benito L, Sangüesa E, Santander S & Zuriaga E (2023) *ACS Biomater. Sci. Eng.* **10(1)**, 178-190.
113. Hinting A & Agustinus A (2020) *Indones. Biomed. J.* **14(2),** 92-98. https://doi.org/10.15562/ijbs.v14i2.230
114. Ferré LB, Kjelland ME, Taiyeb AM, Campos-Chillon F & Ross PJ (2020) *Reprod. Domest. Anim.* **55(6)**, 659-676. https://doi.org/10.1111/rda.13667
115. Huang J, Guo J, Zhou L, Zheng G, Cao J, Li Z, Zhou Z, Lei Q, Brinker CJ & Zhu W (2021) *ACS Appl. Bio Mater.* **4(4)**, 2996-3014. https://doi.org/10.1021/acsabm.1c00105
116. Xie J, Ekpo MD, Xiao J, Zhao H, Bai X, Liang Y, Zhao G, Liu D & Tan S (2022) *Front. Pharmacol.* **13**, 907943. https://doi.org/10.3389/fphar.2022.907943
117. Sharma A, Bischof JC & Finger EB (2021) *Regen. Eng. Transl. Med.* **7**, 57-65. https://doi.org/10.1007/s40883-019-00131-4
118. Hunter H, Getreu N, Wood M & Fuller B (2020) *J Assist Reprod Genet.* **2020**, 505-514.
119. Bunea AI & Taboryski R (2020) *Micromachines* **11(12),** 1048. https://doi.org/10.3390/mi11121048
120. Linkova DD, Rubtsova YP & Egorikhina MN (2022) *Cells* **11(17)**, 2691. https://doi.org/10.3390/cells11172691
121. Nikmahzar A, Khadivi F, Abbasi M, Mahdavinezhad F, Abbasi Y & Daneshi E (2023) *Reprod. Sci.* **30(6),** 1687-700. https://doi.org/10.1007/s43032-022-01113-8
122. Ma Y, Gao L, Tian Y, Chen P, Yang J & Zhang L (2021) *Acta Biomater.* **131**, 97-116. https://doi.org/10.1016/j.actbio.2021.07.001
123. Parihar A, Kumar A, Panda U, Khan R, Parihar DS & Khan R (2023) *Advanced Biology* **7(6),** 2200285. https://doi.org/10.1002/adbi.202200285