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Oocyte cryopreservation
Oocyte assessment and strategies for improving survival

Research Project
Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of B.A or BSc. in Biology

By:
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DEDICATION

This work is dedicated to:

My parents who taught me to follow the path of truth, honesty, justice.

To my siblings, collages and friends.

To all women's on the earth.

Abstract

The procedure known as "oocyte cryopreservation" (OC) involves stimulating ovarian follicles, retrieving the follicular fluid, and isolating and vitrifying mature oocytes. Since 1986, when the first successful pregnancy using cryopreserved oocytes was achieved, OC has been used more frequently as a potential biologic child option for patients undergoing gonadotoxic therapies, such as those used to treat cancer. Planned OC, also known as elective OC, is becoming more and more popular as a way to avoid the age-related decline in fertility. We discuss both planned and medically necessary OC in this narrative review, with an emphasis on the principles of cryopreservation of follicular loss, OC technique, when OC should be done, related financial considerations, and results**.**

Contents

Introduction

Marriage rates are falling and single living rates are rising all throughout the world. The percentage of women who reach their late forties without ever marrying was compiled by the United Nations report as evidence (and it is rising). In the world, is 4.3%.

Because both the quantity and quality of eggs decline with age, fertility declines, especially after the age of 35 (Walker, 2013). From the time a woman reaches puberty until menopause, Getting pregnant occurs when the egg and sperm bind inside the womb. While it might appear that the body is producing a new egg every month, this is not the case. All of a woman's eggs are present at birth and are kept in the ovaries. Eggs age as well with age. Over fifty percent of oocytes died as a result of the aneuploidy rate in oocytes dramatically rising, especially in women over the age of forty (barrett, 2019).

Over 200,000 reproductive-age individuals are diagnosed with cancer each year in the United States Presuming an equal ratio of males to females (Friedler, 1988). The number of new cases in Iraq in 2020, females, all ages is about 19,803 (Lancet, 2019). Cancer is treated with chemotherapy and radiation therapy by eliminating cancer cells. Despite the fact that chemotherapy and radiation are meant to kill cancer cells while protecting healthy tissue, sometimes these therapies harm or kill normal cells. Side effects could result from damage to healthy cells. Chemotherapy can lower estrogen levels, leading to illnesses including primary ovarian insufficiency and symptoms like vaginal dryness. This syndrome causes the ovaries to stop generating eggs early—before the age of 40 (Gook, 2013). Radiation aimed at your pelvis can damage your ovaries and disrupt the creation of estrogen and eggs. The glands that regulate sex and pregnancy hormones can become inactive as a result of radiation therapy administered to the head (Yang, 2018).

Table 1: lists various oncologic treatment regimens and their relative gonadotoxicity risk.

Gonadotoxicity Risk	Treatment/Regimen
High	• Conditioning chemotherapy for bone marrow transplantation • Total body irradiation • Alkylating agents • Pelvic radiotherapy • Brachytherapy for cervical cancer
Intermediate	• Escalated therapy (e.g., BEACOPP) for Hodgkin's lymphoma • Adjuvant chemotherapy agents for breast cancer • Anti-metabolites
Low	• Vinca alkaloids • Anthracyclines • Topoisomerase inhibitors

Since the first child born through *in vitro* fertilization (IVF), it has been more than 40 years. Since then, the field of assisted reproductive technology (ART) has grown significantly, with over 8 million children born globally as a result of IVF (Fauser, 2019). The Society for Assisted Reproductive Technology (SART) National Summary Report's most recent complete data indicates that in 2019, there were almost 300,000 ART cycles performed in the United States, of which nearly 16,000 were for oocyte cryopreservation (OC) (Nikiforov, 2023). Since 1986, when the first successful pregnancy using cryopreserved oocytes took place (Lancet, 2019). Cryopreserved oocytes are now a practical option for future fertility in both medical (e.g., cancer) and planned (e.g., delayed childbearing) indications. This is due to a significant advancement in laboratory techniques (Rienzi & Gracia, 2017). In many branches of biology and clinical medicine, the ability to cryopreserve and store biological cells' and tissues' structure and function is crucial. Early on, the importance of cryopreservation in human assisted reproduction was understood. stage of the technology's development and has increased in due to a variety of clinical, ethical, and legal considerations. Cryopreserved early stage embryos are essential for the effective and secure management of human infertility and subfertility using IVF. However, the ability to cryopreserve human gametes is necessary to postpone conception and/or implantation in situations where embryo cryopreservation is not an option because there isn't a male

or female partner immediately available. First reported in the 1950s, the use of human cryopreserved semen has been successfully used to establish pregnancies and produce subsequent live births in a variety of clinical settings. ` Despite sporadic early successes using human cryopreserved oocytes being reported. Before many of these concerns were allayed by fundamental validation studies conducted in the 1990s, this technology was widely believed to be extremely inefficient and subject to serious safety concerns. As a result, oocyte cryopreservation became popular as a clinical tool, especially in Italy, where a planned legislative change aimed to make it illegal to freeze embryos (Bernard, 2019).

Aim of the study

In order to raise awareness of society in general and those who aspire to motherhood in particular, this research delves deeper into understanding the process with all of its positives and negatives. It also studies the revolutionary opportunity for women to practice motherhood at an advanced age, maintain images of survival, and control their reproductive future.

2. Oocyte Cryopreservation

2.1 Principles of cryopreservation

Living cells must be placed in a state of suspended animation, where they can stay indefinitely and eventually be restored to viability, in order to preserve long-term viability following long-term storage. Although a precise threshold value for this temperature is unknown, the temperature of liquid nitrogen, or −196°C, which is typically used to store mammalian cells, seems to be sufficient for these purposes. Water can only exist as a solid at these low temperatures, and no known biological reactions occur (Friedler, 1988).

The only known threat to cryopreserved cells is background radiation-induced DNA damage. Furthermore, it has been calculated that mammalian cells may survive for hundreds or even thousands of years at the typical terrestrial background radiation levels of 0.1 cgy/y . Since human oocytes can only survive at 37°C and cannot undergo any biological activity at -196°C, the most dangerous periods of cryopreservation seem to be when the temperature is dropping to -196°C and then rising to 37°C. Water condenses into ice, a crystalline substance, when it cools below its freezing point. Ice crystals must necessarily occupy a larger volume than the liquid water from which they originated because ice is less dense than liquid water. Intracellular organelles may sustain significant damage as a result of pressure and shearing forces generated by neighboring liquid water volumes inside a cell solidifying and expanding into ice. Therefore, preventing the formation of ice crystals is one of the main objectives of effective cryopreservation (Park, 1987).

Any solutes in the liquid phase of water are removed from the solid as it turns from liquid to ice. The remaining unfrozen solution's freezing point is lowered as a result. Electrolyte and other solute concentrations can rise to extremely high levels as the temperature decreases and the solid form multiplies. A second important objective of effective cryopreservation is to avoid these solution effects, as these concentrations can be highly toxic to intracellular proteins. The solid ice melts and releases free water when it is reheated, which lowers the osmolarity of the surrounding solution. Slow

rewarming increases the risk of free water thawing and recrystallizing, which could result in more damage. Rapid rewarming can cause abrupt drops in extracellular osmotic pressure, which can cause free water to quickly move across and into the cell, causing swelling and damage to the cell. Avoiding osmotic shock is the third main objective of effective cryopreservation (Blerkom, 1994). Consequently, putting oocytes in liquid nitrogen by themselves is not a viable cryopreservation method. Three problems that must be avoided by all successful methods are osmotic shock, solution effects, and ice-crystal formation. Until now, extra chemicals have been used in all cryopreservation strategies, including oocyte cryopreservation techniques, to prevent cell damage. These substances are referred to as cryoprotectants, and they fall into two groups: permeating and nonpermeating (Elsevier, 2006).

2.2 Permeating cryoprotectants

Small molecules known as permeating cryoprotectants easily pass through cell membranes. By creating hydrogen bonds with water molecules, they stop ice from crystallizing. They reduce the freezing point of the resulting mixture at low concentrations in water. Nevertheless, at sufficiently high concentrations (Walker, 2013). They prevent the formation of the distinctive ice crystal and instead cause the formation of a solid, glass-like state known as the vitrified state, in which water solidifies but does not expand. By preventing ice crystals from forming, the permeating cryoprotectants accomplish the primary objective of effective cryopreservation. The composition of permeating cryoprotectants that are widely used (Elsevier, 2006).

2.3 Nonpermeating cryoprotectants

Nonpermeating cryoprotectants stay extracellular, in contrast to permeating cryoprotectants. They work by removing free water from the cell, which causes the intracellular area to become dehydrated. Consequently, their combined application increases the net concentration of the permeating cryoprotectant in the intracellular space of the cryoprotectant. This helps the permeating cryoprotectant even more in its effort to stop the formation of ice crystals. During thawing, the nonpermeating

cryoprotectants are crucial. The water produced by the melting ice quickly lowers the extracellular osmotic pressure during thawing. If the intracellular cryoprotectant does not diffuse out fast enough to stop an excessive amount of free water from entering the cell and causing swelling or even rupture, osmotic shock may result. Consequently, during the thawing stage of freezing and thawing protocols, a high concentration of nonpermeating cryoprotectants is frequently used. While other disaccharides and nonpermeating agents can also be used, sucrose is the most widely used nonpermeating cryoprotectant. Trehalose has been found to have a novel application for nonpermeating cryoprotectants. Trehalose is injected straight into the cell, as opposed to being added to the extracellular solution. It stays intracellular in this way, where it seems to function as a permeating cryoprotectant, preventing the formation of ice crystals during cooling (Bernard, 2019).

2.4 Technique

Follicloid genesis proceeds through the primordial, primary, secondary, preantral, and antral stages in a continuous and stochastic manner. Gonadotropin is not necessary for follicular growth until the preantral and antral stages, at which point it is reliant on luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In a normal menstrual cycle, around 10 antral follicles start with gonadotropin-dependent growth, but gradual lowering of FSH levels during the follicular phase limits the time it is above a critical threshold, ultimately favoring mono-follicular growth (Santbrink, 1995). Typically, only the one follicle with the highest sensitivity to FSH will continue to be stimulated and ovulate (Orisaka et al., 2021). A germinal vesicle (GV) oocyte is an arrest of primordial follicles in prophase I, and meiotic competence is not acquired until shortly before ovulation (Jones, 2019). The oocyte advances to metaphase of meiosis II after the LH surge and becomes arrested there; meiosis is not fully completed until fertilization. The general procedure for oocyte cryopreservation is rather simple and involves a few essential steps (as shown in Figure 1), regardless of the indication: Mature oocytes are cryopreserved after controlled ovarian stimulation (COS) and

oocyte retrieval (since only mature oocytes can be fertilized). Exogenous gonadotropins administered with daily injections during COS effectively prolong the duration of the previously mentioned FSH threshold, enabling multifollicular development. A more thorough analysis of COS is outside the purview of this review, as there are numerous ovulation induction regimens and protocols available to achieve ovarian stimulation. Patients usually need (7–12/) days of ovarian stimulation; during this time, transvaginal ultrasound and serum hormone levels will be used to periodically monitor the patients' progress. Usually, drugs that simulate the natural LH surge are used to "trigger" follicle maturation once (1-2) follicles are measured to be larger than 18 mm. Oocyte retrieval takes place approximately 36 hours following the trigger drug to increase the rates of oocyte maturation while lowering the chance of spontaneous ovulation (Andersen, 1995).

The oocyte retrieval is a less than 30-minute outpatient procedure that involves transvaginal ultrasound guidance to perform needle aspiration of the contents of ovarian follicles. The embryologist isolates and chooses the oocytes within this fluid for cryopreservation. Usually, it takes two weeks to complete the entire process. A DuoStim protocol, in which a second OC cycle is started soon after the first retrieval, can increase the number of mature eggs frozen in patients who are able to postpone gonadotoxic treatment for a longer period of time—roughly four weeks (Vaiarelli, 2018).

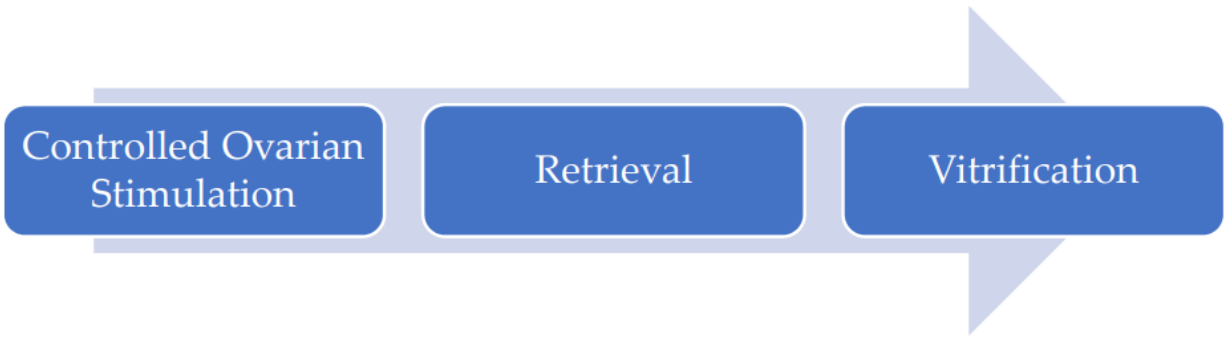

Figure 1: General process of oocyte cryopreservation.

Adnexal torsion, thromboembolism, and ovarian hyperstimulation syndrome (OHSS) are complications associated with ovarian stimulation. Increases in vascular permeability cause internal fluid to move from intravascular to extravascular spaces, which is a characteristic of ovarian hyperstimulation syndrome. Commonly used as the trigger drug, human chorionic gonadotropin (hCG) is believed to play a major role in the pathogenesis by causing the release of vasoactive substances, especially vascular endothelium growth factor (VEG) (Fauser, 2019). The Clinical symptoms can progress to severe/critical stages complicated by oliguria, severe ascites, hemoconcentration, thromboembolism, arrhythmias, pleural effusions, adult respiratory distress syndrome, and/or sepsis. Mild cases of the condition start with abdominal distension, mild nausea, and diarrhea. Mild OHSS characteristics can be found in 20% of IVF cycles, but moderate and severe forms are far less common—less than 5% of cycles have them (Orisaka et al., 2021).

2.5 Evolution of methods applied to oocyte cryopreservation.

2.5.1 Slow cooling

Slow cooling consists of gradual cell dehydration, combining relatively low CPA concentrations (≤1.5 mol/l for P-CPAs and ≤0.3 mol/l for NP-CPAs) with controlled slow cooling rates. The P-CPAs and NP-CPAs are obtained by means of a specifically de- signed machine (programmable freezer), which ensures accurate and consistent cooling parameters throughout the procedure. Oocytes are first equilibrated in solutions containing low P-CPA concentrations (typically 1.5 mol/l): as the cell membrane is more permeable to water than to any CPA, at first, the intracellular water efflux is not counterbalanced by an equivalent influx of P-CPA, so initially resulting in cell shrinkage (Edgar, 2013). Successively, as CPAs enter the cell, the volume is gradually re-expanded.

The subsequent addition of NP-CPA (most frequently sucrose or trehalose, typically ≤0.3 mol/l) restores the osmotic gradient, inducing additional cell dehydration. At last, oocytes are loaded onto specially designed straws and cooled to −8°C (−2°C/min), at

which temperature manual seeding is performed to induce ice nucleation; the formation of ice in the solution allows further dehydration by means of an increase in extracellular solute con- centration. Cooling continues slowly (−0.3°C/min to −1°C/min) to −30°C. During this phase, the growing ice formation removes most of the water in the solution, solute concentration further increases and an amorphous solidification state is reached (Fauser, 2019).

The temperature is then rapidly reduced to −150°C (−50°C/min) before the final plunge and storage in liquid nitrogen at −196°C. Thawing is performed rap- idly and proceeds in a mirror-like fashion with respect to cooling; oocytes are exposed to solutions with decreasing concentrations of NP-CPAs to obtain a stepwise gradual rehydration (Rienzi & Gracia, 2017).

2.5.2 Vitrification

A significant development in IVF was the more recent emergence of vitrification. Currently, Kuwayama and colleagues have described the most widely used protocol for oocyte vitrification:It is a two-step process that involves adding CPAs to cryo-media step-by-step (Gook, 2013). The oocytes are transferred to a vitrification solution containing 15% v/v ethylene glycol and 15% v/v DMSO, along with 0.5 mol/l sucrose, following a first equilibration phase of (5–15) min in a solution containing 7.5% v/v ethylene glycol and 7.5% v/v DMSO. Following a brief incubation (up to one minute) in the vitrification medium, the sample is moved onto specially made apparatuses and ultimately submerged in liquid nitrogen at -196°C, where it is kept until needed. Vitrification has been done on several cryosupports over the years; these have been previously reviewed.

Following the initial encounter with conventional straws (0.25 ml), which demonstrated their unsuitability, it was recommended to utilize new devices that ensure minimal volume loading (1 µl). Actually, because of the diminished thermoinsulator effect, the lower contact surface between the sample and liquid nitrogen enabled extraordinarily high cooling rates. Since then, many other cryo-tools have been proposed, but as of right now, the majority of centers worldwide probably prefer

supports made of a thin plastic filmstrip that is attached to a holder, covered by a tubular cap, and not further sealed (Vaiarelli, 2018). Numerous other "open systems," such as capillaries, nylon loops, and modified straws, have been effectively employed to ensure extremely rapid direct contact with liquid nitrogen. Over time, scientists have developed innovative closed systems to improve protection against the risk of disease transmission. This is because the liquid nitrogen itself can be contaminated by multiple pathogens and the loaded samples may be infected, making it a vector for potential cross-contamination. Closed devices offer a physical barrier that separates the sample from liquid nitrogen, thereby offering a theoretically higher level of biosafety compared to open devices. Closed systems, on the other hand, have been found to typically have lower rates of warming and cooling, which may have an adverse effect on the final laboratory and clinical results. Furthermore, there have been no cases of disease transmission in the history of cryotransfers using assisted reproductive technology, although there is much discussion about whether closed or open systems are preferable (Rienzi L. , 2017).

Alternative strategies to reduce the risk of cross-contamination during cryopreservation include: (a) using sterile air; (b) storing liquid nitrogen in its vapor phase, which may contain a lower density of environmental airborne contaminants; and (c) using sterilized liquid nitrogen via ultraviolet light. To prevent the formation of ice crystals during the transition state, heating is done quickly. The support is immersed in either a prewarmed warming solution with a high sucrose concentration (1 mol/l) or in a water bath (in the case of closed systems). (If open gadgets are utilized). Subsequently, the CPAs are eliminated gradually, with cooling occurring in a manner akin to a mirror. Following their last washing, the oocytes are then cultured in a suitable medium until they are needed (Table 2).

In the recent years, numerous studies have demonstrated the superiority of vitrification over slow-freezing protocols and its non-inferiority when compared to fresh cycles, ever since the first reported success with human oocytes (barrett, 2019).

	Slow cooling	Vitrification
Equilibrium freezing	Yes	No
Concentration of cryoprotectants	Low	High (potential toxicity)
Ice crystal formation	Yes	No
Programmable freezing machine	Yes	No
Cooling rates	0.3°C/min to minimize ice crystal formation	20 000°C/min to generate a glass-like structure (no crystallization)
Dependence on operator's skills	Limited	High. Specific training needed (learning curve)
Reproducibility of the results	High	Variable across different operators
Complexity of cell manipulation	Low	High due to cryoprotectant viscosity
Protocol deviation	Possible	Limited
Time-consuming	Yes	No

Table 2: Comparison between slow cooling and vitrification protocols

CONCLUSION

Oocyte cryopreservation is a key technique in any IVF program, finally coming to complete fruition thanks to the notable achievements of the last decades. According to the available evidence, vitrification is considered the reference standard in oocyte freezing, leading to striking improvements in final outcomes and broadening its clinical application. Currently, it is a viable option for infertile patients as well as for women who want to preserve their fertility, both for medical or nonmedical reasons, and its benefits are already visible. However, long-term follow-up studies on children born from frozen oocytes are still lacking, and further research is needed. Finally, future efforts should focus on standardization and/or automation of the technique to enhance its consistency and efficiency.

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