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Direktor: Universitätsprofessor Dr. med. P. K. Mallmann

RNA-Seq Analysis Identifies Transcriptomic Alterations in Optimized Cryopreservation Protocols for Human Ovarian Tissue

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1. Gutachterin: Professorin Dr. med. G. Rahimi
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List of abbreviations

DMSO: dimethyl sulfoxide
EG: ethylene glycol
PrOH: propanediol
3D: three-dimensional
HAS: human serum albumin
ROS: reactive oxygen species
HPO: hypothalamic-pituitary-ovarian axis
GnRH: gonadotropin-releasing hormone
FSH: follicle-stimulating hormone
LH: luteinizing hormone
GSH: glutathione
OHSS: ovarian hyperstimulation syndrome
CPAs: cryoprotectants
AFPs: antifreeze proteins

1. Zusammenfassung

Zusammenfassend ergab diese Metaanalyse, dass konventionelle langsame Kryokonservierung und Vitrifikation vergleichbare Ergebnisse hinsichtlich der Follikellebensfähigkeit, des Anteils intakter Primordialfollikel, des Anteils DNA-fragmentierter Follikel und des Anteils Stromazellen zu liefern scheinen. Die eingeschlossenen Studien unterschieden sich jedoch in der Anwendung der Kryokonservierungsprotokolle. Daher sind weitere Studien erforderlich, um die optimale Methode für die Kryokonservierung menschlichen Eierstockgewebes zu bestimmen. Obwohl hinsichtlich der Anatomie des Eierstockgewebes kein signifikanter Unterschied zwischen traditioneller Kryokonservierung und Vitrifikation besteht, hat jede Methode ihre eigenen Vorteile und eignet sich für unterschiedliche klinische Szenarien. Zu den Vorteilen der traditionellen Kryokonservierung zählen ihre ausgereifte Technologie und relativ standardisierte Verfahren. Die Vitrifikation hingegen bietet Vorteile wie schnelles Einfrieren, einfache Handhabung und hohe Flexibilität. Bislang gilt die Kryokonservierung von Eierstockgewebe als experimentelles Verfahren. Die Wahl der Methode kann an spezifische klinische Bedürfnisse angepasst werden.

Die Kryokonservierung von Eierstockgewebe mit der beschriebenen Methode verringert nicht die Follikelproduktion, sondern führt zu einer Herunterregulierung der ovariellen Steroidogenese und damit zu einer Verringerung der Östrogen- und Progesteronsekretion. Das schnelle Auftauen von Eierstockgewebe erhöht die Proliferation und Apoptose der Zellen.

2. Introduction

With the advancement of medical technology and the increasing awareness of social issues, fertility protection is gradually gaining more attention and importance. There are currently three mainstream ways to protect female fertility: oocyte cryopreservation, embryo cryopreservation, and ovarian tissue cryopreservation[1,2].

Oocyte cryopreservation is one of the most widely used methods of fertility preservation. For oocyte preservation, a woman undergoes controlled ovarian hyperstimulation with injectable gonadotropins to recruit multiple follicles. Her response to the medications is monitored through frequent ultrasounds, typically every 1–2 days, and by measuring serum hormone levels, allowing therapy to be tailored for optimal outcomes. Once the follicles reach the appropriate size, maturation is triggered using an injection of human chorionic gonadotropin (hCG), a gonadotropin-releasing hormone (GnRH) agonist, or a combination of both. Approximately 36 hours later, oocytes are retrieved transvaginally under ultrasound guidance. At this stage, the oocytes can be frozen for future use[3,4].

After the oocytes are taken out, we will freeze them. The main methods of cryopreservation are slow freezing and vitrification. The slow-freeze method utilizes low initial concentrations of cryoprotectants, which reduces toxicity while the oocyte is still metabolically active. Since cellular metabolism is estimated to decrease by approximately 50% for every 10°C drop in temperature, the potential for toxicity is minimized by increasing the concentrations of cryoprotectants and other solutes only after the cell has been cooled to temperatures at which metabolic activity is significantly reduced. At approximately −6°C, ice crystal formation can be initiated in the solution through a process known as seeding. This involves introducing a small ice crystal, which acts as a nucleus to promote crystallization of surrounding water molecules. Seeding is typically performed by briefly touching the exterior of the cryopreservation vessel with a very cold instrument, such as forceps, thereby inducing the formation of an initial ice crystal at the point of contact [5,6].

The other method is vitrification. During vitrification, permeating cryoprotectants are introduced at high concentrations while the oocyte is maintained at room temperature. Due to the substantial toxicity associated with these concentrations, the oocyte can only be exposed for a very brief equilibration period before being plunged directly into liquid nitrogen. To further prevent ice crystal formation, an extremely rapid cooling rate is employed. Consequently, novel cryovessels have been developed that enable direct contact between the oocyte-containing solution and liquid nitrogen, characterized by a very high surface-to-volume ratio. With

advances in the understanding of cryoprotectants, vitrification has become a highly refined technique and is now increasingly recognized as the preferred method for oocyte cryopreservation[7,8]. Although the technology is relatively advanced, its effectiveness is still significantly influenced by age. Clinical pregnancy rates per thawed oocyte range from 4.5-12%, depending on age [9]. Studies also have shown that cryopreserving oocytes at a younger age increases the likelihood of successful outcomes [2,10].

Several key parameters are considered in oocyte cryopreservation, including cell characteristics, membrane permeability to cryoprotectants, cryoprotectant toxicity, as well as the temperature and duration of exposure. Cryogenic injury remains the principal barrier to successful oocyte cryopreservation [11-14]. The damage has been shown to affect cell membranes, microtubules, cytoskeletal structures, and the zona pellucida. Chromosomal abnormalities have also been observed following cryopreservation. The most striking effect is observed in the second meiotic spindle, where microtubules are disrupted or disassembled due to tubulin depolymerization. In mouse oocytes, spindle abnormalities can appear after just 10 minutes of exposure to 25°C[14]. Similarly, meiotic spindles in human oocytes are highly sensitive to cold, with incubation at both 25°C and 4°C significantly impairing spindle assembly[15].

Several strategies have been developed to mitigate cold-induced damage. Notable improvements have been achieved through rapid cooling during the transition phase and by supplementing oocytes with agents that stabilize the plasma membrane. Adding non-permeable cryoprotectants is a common and effective method. Non-osmotic cryoprotectants, such as sucrose or trehalose, primarily play an extracellular protective role in oocyte freezing. They regulate extracellular osmotic pressure, allowing water to be expelled from the cell, thereby reducing intracellular ice crystal formation. They can also form a protective layer outside the cell membrane, which can stabilize the membrane structure and reduce mechanical and osmotic stress caused by freezing/thawing. Moreover, increasing sucrose concentration in the freezing medium has been shown to enhance preservation of the chromosome segregation apparatus, with retention rates approaching those of freshly collected oocytes [16,17]. Structural and membrane protectants are commonly added to enhance the success of oocyte cryopreservation. Examples include cholesterol, cholesterol inclusion complexes, and serum albumin. These agents help stabilize the plasma membrane, improve membrane elasticity, and buffer osmotic stress, thereby indirectly protecting the cytoskeleton and chromosomes [18,19]. During the freezing and thawing process of oocytes, low temperature stress and osmotic pressure changes can lead to excessive production of reactive oxygen species (ROS). Excessive production of ROS can damage oocytes by inducing membrane

lipid peroxidation, mitochondrial dysfunction, and chromosomal abnormalities, ultimately triggering apoptosis and reducing both oocyte quality and survival rates [20-22]. Antioxidants play a protective role during oocyte cryopreservation by scavenging reactive oxygen species, preserving mitochondrial function and membrane integrity, and stabilizing chromosome structure. Their use has been shown to improve post-thaw oocyte survival rates and developmental potential. Glutathione (GSH), one of the most widely used antioxidants in oocyte cryopreservation, enhances post-thaw oocyte quality and developmental potential by scavenging reactive oxygen species, preserving mitochondrial function, and stabilizing chromosomes. It is commonly supplemented directly into freezing, thawing, or culture media, and intracellular levels can also be elevated through precursor compounds. GSH thus serves as a critical protective factor during oocyte cryopreservation[22-24].

Embryo cryopreservation occurs after the oocytes are removed, the oocytes are inseminated with sperm, and the resultant embryos are cultured and frozen. The study indicated that the survival rate of each thawed embryo is 35-90%, the implantation rate is 8-30%, and the cumulative pregnancy rate is over 60% [25]. During an oocyte cryopreservation cycle, the use of hCG as an ovulation trigger may still lead to ovarian hyperstimulation syndrome (OHSS) in some patients, particularly those with a high ovarian reserve or polycystic ovary syndrome. This occurs because hCG stimulates the corpus luteum to secrete large amounts of vasoactive substances, such as VEGF, which increase vascular permeability and cause fluid to leak into the peritoneal or thoracic cavities. As a result, patients may experience severe complications, including abdominal distension, ascites, hemoconcentration, and thrombosis [4,26]. Embryo cryopreservation can effectively prevent OHSS, related to hCG secretion during early pregnancy.

Ovarian tissue cryopreservation has become a critical strategy for preserving female fertility and maintaining ovarian endocrine function[27]. Unlike oocyte cryopreservation and embryo cryopreservation, it is currently the only fertility preservation option available for prepubertal girls[28-30]. Moreover, ovarian tissue cryopreservation circumvents the time constraints associated with oocyte or embryo freezing, which require several weeks of hormonal stimulation—an impractical option for patients with malignancies needing urgent treatment. By enabling immediate preservation without delaying therapy, ovarian tissue cryopreservation offers a vital solution for fertility preservation[31]. Additionally, because it retains both oocytes and stromal tissue, this approach can help restore endocrine function, presenting significant promise for women at risk of premature ovarian failure[32,33]. Therefore, it is essential to explore further and optimize ovarian tissue cryopreservation techniques to enhance clinical outcomes.

The primary process of ovarian tissue cryopreservation involves placing the ovarian tissue in a cryoprotectant solution immediately after procurement, followed by the removal of the ovarian medulla to prevent ice crystal formation during the freezing process, which can result in cellular damage. The tissue is then frozen using various cryopreservation techniques. The two main cryopreservation methods currently utilized are traditional slow freezing and vitrification[34,35].

Traditional slow freezing is characterized by controlled freezing, typically regulated by a computer program, where the tissue is gradually frozen in the cryoprotectant solution. This method minimizes ice crystal formation through precise control of the freezing rate, thereby reducing tissue damage. As a result, slow freezing remains the most widely employed technique for ovarian tissue cryopreservation. However, it requires specialized equipment and is time-consuming[36,37].

Traditional slow freezing is one of the earliest methods used for the preservation of germ cells and ovarian tissue. After decades of clinical application, the laboratory technology and operation process are mature [38-40]. During the freezing process of ovarian cortical fragments, slow freezing allows precise control of the temperature drop rate through a programmable cooling device, ensuring uniform water migration within and outside the tissue, reducing local ice crystal formation, and thus preserving the integrity of the follicle structure. Clinically, several patients with malignant tumors have successfully recovered endocrine function and achieved natural pregnancy after thawing through the use of slow freezing to preserve ovarian tissue[41-43]. Traditional slow freezing technology is mature and relatively controllable during the cooling process. In the event of an instrument malfunction and temperature fluctuations, laboratory personnel can immediately pause the cooling program or manually adjust the program to return the temperature to the preset curve, preventing sudden frostbite of the ovarian tissue. In the event of insufficient protectant penetration, laboratory personnel can extend the protectant equilibration time or adjust the protectant concentration to fully dehydrate the cells and reduce the risk of ice crystal formation. In the event of operational delays or abnormal liquid nitrogen storage, laboratory personnel can quickly replenish liquid nitrogen and move temperature-sensitive samples to spare tanks to ensure that the tissues remain at a safe temperature. Experienced laboratory personnel can adjust the protocol promptly to minimize freezing damage. At the same time, they can monitor the temperature curve throughout the slow freezing process to ensure that the ovarian tissue is cryopreserved according to the programmed cooling rate throughout the process, which is a key advantage of slow freezing[44,45].

At the same time, traditional slow freezing has more advantages in freezing large pieces of tissue. First, large pieces of tissue are large and have uneven water distribution. Traditional slow freezing allows the water in the cells to slowly migrate to the outside of the cells, reducing the formation of ice crystals in the cells. Secondly, the thickness of large pieces of tissue is usually uneven. The temperature control program of slow freezing can adjust the cooling rate according to the size of the tissue to ensure that the temperature of the internal core and the outer layer drops synchronously, reducing local supercooling or local ice crystals. This slow, controlled freezing process can reduce the risk of mechanical damage and protect the integrity of the follicle and matrix structure. For large pieces of tissue, high concentrations of protective agents are difficult to penetrate and are prone to toxicity. Traditional slow freezing can provide protection using lower concentrations of permeable protective agents, reducing the impact of chemical toxicity on the tissue.

Although traditional slow freezing of ovarian tissue is a well-established technique that effectively preserves tissue structure, it has several limitations. The procedure is labor-intensive, requiring specialized operator training, dedicated cryopreservation equipment, precise parameter settings, and regular maintenance. Additionally, the process is time-consuming: preparation involves cryoprotectant penetration and pre-cooling, followed by a slow freezing stage at approximately −0.3°C per minute down to −34°C, and subsequent cooling to −140°C, with total processing taking at least four hours before storage in liquid nitrogen. The method is also costly, due to equipment purchase, maintenance, and personnel training. Moreover, in clinical situations where rapid preservation is needed, traditional slow freezing may not provide timely or effective cryopreservation [46].

In contrast, vitrification is an ultra-rapid cryopreservation method that utilizes high concentrations of cryoprotectants to freeze the tissue rapidly in liquid nitrogen, forming an amorphous glass-like solid and preventing ice crystal formation. The procedure begins with slicing the ovarian cortex into 1–2 mm sections, followed by thorough washing. The tissue is then exposed to a combination of permeable cryoprotectants and non-permeable cryoprotectants using either a stepwise or short-term protocol to partially dehydrate intra- and extracellular water and minimize ice crystal formation. The prepared ovarian slices are subsequently rapidly cooled to −196°C in liquid nitrogen within seconds for long-term storage[47,48].

In ovarian tissue vitrification, the selection of cryoprotectants (CPAs) is critical for tissue survival and successful transplantation. Cryoprotectants play an important role in minimizing ice crystal formation within cells during the cryopreservation process[49]. The type, concentration,

and combination of cryoprotectants are critical in ensuring that the tissue can effectively navigate the two "high-risk" phases: freezing and thawing. The selection of an appropriate cryoprotectant is central to enhancing the quality of ovarian tissue cryopreservation[50,51]. Cryoprotectants can be classified into two categories: permeable and non-permeable.

Permeable cryoprotectants: Common examples include dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propanediol (PrOH). They penetrate cells, displace water, and substantially reduce ice crystal formation, thereby protecting follicle membranes, stromal cells, and organelles. However, excessively high concentrations can be cytotoxic, damaging cell membranes and mitochondria, while insufficient concentrations may fail to prevent ice crystal formation. These small molecules are capable of penetrating the cell membrane, thereby preventing intracellular ice formation during freezing. However, the concentration of permeable cryoprotectants must be carefully controlled, as excessively high concentrations can lead to cytotoxicity, while too low a concentration may fail to prevent ice crystal formation[52,53].EG is the most commonly used cryoprotectant in human ovarian tissue cryopreservation and is frequently combined with other CPAs, polymers, and proteins to enhance tissue protection[54-57]. Research has shown that combinations of DMSO and EG are particularly effective as permeable cryoprotectants due to their synergistic effect in preserving cell integrity[51].

Non-permeable CPAs, such as sucrose, lactose, and sorbitol, regulate extracellular osmotic pressure, promote cell dehydration, mitigate cell volume changes, and reduce the required concentration of permeable CPAs, thereby minimizing toxicity. Unlike permeable agents, sucrose does not enter the cell but primarily acts by regulating the osmotic pressure outside the cell. This helps reduce cell rupture and dehydration during the freezing process[58]. Clinical and experimental studies have shown that the optimal combination of permeable and non-permeable CPAs not only limits ice crystal damage but also enhances follicle survival, post-thaw ovarian function, and transplantation outcomes[51]. The combination of DMSO, EG, and sucrose is often employed in experiments, as it effectively balances osmotic pressure and minimizes ice crystal formation, thereby improving the overall preservation of the ovarian tissue. Through careful optimization of cryoprotectant types and concentrations, the cryopreservation method for ovarian tissue can be significantly improved, enhancing the viability and functionality of the preserved tissue.

In addition to conventional cryoprotectants, antifreeze proteins (AFPs) have recently emerged as a novel class of biological cryoprotectants widely studied for the cryopreservation of ovarian tissue, oocytes, and embryos. AFPs were first identified in polar fish and later discovered in insects, plants, and microorganisms. These proteins exhibit unique biochemical properties that

function through two primary mechanisms under low-temperature conditions: binding to ice crystal surfaces to inhibit crystal growth and recrystallization, and regulating the thermal hysteresis gap between the freezing and melting points, thereby delaying ice crystal formation and maintaining extracellular ice crystals in a small and stable state [59-61]. Additionally, AFPs can associate with cell membranes, reducing low–temperature–induced lipid phase transitions and changes in membrane permeability, thereby indirectly protecting cellular structures and mitochondrial function[62,63].

Experimental studies have demonstrated that the incorporation of various AFPs into ovarian tissue cryopreservation protocols improves post-thaw tissue viability and follicular structural integrity. Subsequent animal studies have shown high proportions of morphologically normal follicles after AFP-assisted vitrification—approximately 60% in mice, 70% in rabbits, and 70% in cows [64-66]. In cattle, the cryoprotective effect of these substances was observed for the first time, which improved the morphological rate (70%) and reduced the level of follicular apoptosis (24%)[66]. Collectively, these findings highlight the promising role of AFPs as effective cryoprotective agents in ovarian tissue vitrification.

While vitrification is faster and simpler, the use of high cryoprotectant concentrations can lead to cytotoxicity and tissue damage. Furthermore, the rapid freezing process may not allow uniform temperature stabilization throughout the tissue, potentially causing thermal gradients that can result in tissue damage, particularly in larger tissue volumes[46,67].

Despite the advantages of both methods, there remains ongoing debate regarding their comparative efficacy and safety, necessitating further investigation. This study will conduct a systematic review and meta-analysis to evaluate and compare the two cryopreservation methods.

Thawing is another critical step in the cryopreservation process. Before transplantation, frozen ovarian tissue must be gradually warmed to physiological temperatures using specialized heating devices to prevent ice crystal formation. Improper thawing can lead to intracellular ice formation and subsequent tissue damage. This study will also explore the impact of varying thawing rates on the preservation and recovery of human ovarian tissue, shedding light on the optimization of thawing protocols for enhanced tissue viability.

To optimize the cryopreservation method for ovarian tissue, it is essential to investigate the key factors influencing the success of cryopreservation. These factors primarily include the following:

1) Cryoprotectants

During the cryopreservation and thawing process of ovarian tissue, CPAs not only prevent ice crystal formation during the cooling phase but also play a critical protective role during the thawing phase. During the thawing process, tissue rapidly warms from extremely low temperatures to room temperature. Without proper CPA protection, cells are prone to ice recrystallization, osmotic imbalance, and membrane rupture, leading to follicular death or tissue damage.

During post-thaw CPA removal, a stepwise dilution or short, step-wise elution process is used to gradually reduce the CPA concentration to prevent cell rupture due to rapid changes in osmotic pressure. The primary role of CPA during this phase is to buffer osmotic stress, maintain cell membrane integrity, and reduce oxidative stress caused by freeze-thaw. Studies have shown that properly controlling the type, concentration, and removal rate of CPA can significantly improve post-thaw follicular cell survival and the recovery of endocrine function in ovarian tissue [68].

2) Thawing rate

Thawing is a critical step in restoring cryopreserved ovarian tissue to its viable state by bringing the tissue back to room temperature. The optimization of the thawing process, particularly by controlling the thawing rate, is essential to minimizing ice crystal formation within the cells, preserving osmotic balance between the inside and outside of cells, and reducing cellular damage.

The slow thawing method typically involves immersing the cryotube in a 37℃water bath for approximately 1 minute until the tissue is completely thawed. In contrast, the quick thawing method utilizes a 100℃ (boiling water) water bath. The exposure time to the boiling water is visually controlled by monitoring the presence of ice within the culture medium. Once the ice reaches a size of approximately 2 to 1 mm, the sample bottle is removed from the boiling water. At this stage, the final temperature of the culture medium generally falls between 4℃ and 10℃ [69]. The quick thawing method is aligned with the principles of cryobiology, which suggest that any organism or tissue cryopreserved using current techniques should be thawed as quickly as possible. This quick thawing helps minimize the exposure of cells to cryoprotectants, thereby reducing excessive dehydration and osmotic imbalances that could lead to cell damage[70,71]. However, more study about this method is needed.

This study will show the positive effect of elevated thawing rate for cryopreservation of human ovarian tissue in the following text.

3) In vitro culture

In vitro culture of ovarian tissue following cryopreservation has become a critical strategy to enhance the success rate of ovarian tissue transplantation and optimize the recovery of tissue function. Several methods are commonly employed for in vitro culture, including single follicle separation culture, tissue block culture, and three-dimensional (3D) scaffold culture[72].

Single follicle separation culture involves isolating individual follicles from the ovarian tissue and culturing them independently. While this method allows for the culture of follicles, it lacks the natural ovarian tissue microenvironment that is necessary for proper follicular development and function[73]. Tissue block Culture involves culturing the ovarian tissue in block form, which retains the natural ovarian tissue microenvironment. However, a limitation of this method is the potential for hypoxia and necrosis in the center of the tissue block due to insufficient nutrient and oxygen supply. 3D scaffold culture provides a 3D structural support that closely mimics the ovarian stroma, offering superior support for follicle development. This method is considered an effective approach for in vitro culture, as it promotes better follicular growth and maturation by simulating the natural 3D structure of the ovarian tissue. The selection of appropriate 3D scaffold materials is critical for the success of this method.

In conclusion, while various strategies for female fertility preservation possess distinct strengths and limitations, the refinement of human ovarian tissue cryopreservation protocols remains a critical focus of reproductive medicine. Ongoing research aimed at optimizing cryoprotectant formulations, cooling methods, and post-thaw tissue viability assessments will be essential for enhancing the efficiency and safety of cryopreservation. Continued progress in this field will not only advance the preservation of female fertility but also provide valuable experimental models for improving reproductive outcomes and expanding clinical applications in fertility preservation.

This study aims to evaluate the differential effects of various freezing methods on ovarian tissue cryopreservation and to systematically investigate how different thawing protocols influence tissue integrity and cellular physiological function. Through RNA sequencing, a comprehensive transcriptomic analysis of cryopreserved ovarian tissues will be conducted to elucidate changes in gene expression profiles. By identifying alterations in key regulatory genes and uncovering their potential biological significance, this research seeks to provide a scientific foundation for optimizing ovarian tissue cryopreservation and culture protocols. The findings are expected to offer novel insights into the molecular mechanisms underlying

cryopreservation-induced changes and contribute to the advancement of fertility preservation technologies, ultimately improving treatment options for patients at risk of fertility loss.

3. Comparison of the quality of ovarian tissue cryopreservation by conventional slow cryopreservation and vitrification ——a systematic review and meta-analysis

Qingduo Kong[1] , Cheng Pei[1], Gohar Rahimi[1,2], Peter Mallmann[1] and Volodimir Isachenko[1*]

1. Department of Obstetrics and Gynecology, Medical Faculty, Cologne University, 50931 Cologne, Germany.
2. Medizinisches Versorgungszentrum AMEDES für IVF- und Pränatalmedizin in Köln GmbH, 50968 Cologne, Germany.

*Correspondence: vladimir.isachenko@uk-koeln.de

3.1. Abstract

Background: Ovarian tissue cryopreservation is increasingly applied in patients undergoing gonadotoxic radiotherapy or chemotherapy treatment or other patients who need to preserve their fertility. However, there is currently limited evidence to know which type of ovarian tissue cryopreservation is better. The advantages and disadvantages of conventional slow cryopreservation and vitrification are still controversial. The purpose of this meta-analysis was to analyze the ovarian tissue quality of ovarian tissue cryopreservation by conventional slow cryopreservation and vitrification.

Methods: According to the keywords, Pubmed, Embase, and Cochrane Library were searched for studies to January 2024. Studies comparing the follicular viability of conventional slow cryopreservation versus vitrification were assessed for eligibility. The meta-analysis was performed using Stata software (Version 12.0) and Review Manager (Version 5.2).

Results: A total of 18 studies were included in this meta-analysis. The pooled results of the primary outcomes indicated that there was no difference between the two approaches for follicular viability (RR = 0.96, 95% CI: 0.84–1.09, P=0.520, I^2=95.8%, Random-effect), the proportion of intact primordial follicles (RR=1.01, 95% CI: 0.94–1.09, P=0.778, I^2=70.6%, Random-effect). The pooled results of the secondary outcomes indicated that there was no difference between the two approaches for the proportion of DNA fragmented follicles (RR=1.20, 95% CI: 0.94–1.54, P=0.151, I^2=0.0%, Fixed-effect), and the proportion of stromal cells (RR=0.58, 95% CI: 0.20–1.65, P=0.303, I^2=99.7%, Random-effect).

Conclusions: Conventional slow cryopreservation and vitrification appear to provide comparable outcomes. The heterogeneity of the literature prevents us from comparing these two techniques. Further high-quality studies are needed to enhance this statement. This meta-analysis provides limited data which may help clinicians when counselling patients.

Keywords:

Cryopreservation; Vitrification; Ovary or Ovarian Tissue; Ovarian Follicles

3.2. Introduction

Ovarian tissue cryopreservation has become a widely adopted and significant method for fertility preservation as an increasing number of women become aware of its benefits(1-4). Compared with oocyte cryopreservation, ovarian tissue cryopreservation is the only option for preserving fertility in women undergoing cancer treatment that cannot be delayed or in prepubertal girls for whom mature germ cells are not available(5-10). Additionally, ovarian cryopreservation can also be applied to patients with ovarian benign tumors that require ovarian removal and premature ovarian failure. Advancements in autologous transplantation technology following ovarian cryopreservation are also making significant strides, enabling the restoration of endocrine function. (11-15).

Since the birth of the first baby using ovarian tissue cryopreservation in 2004, more than 200 babies have been born worldwide through this technology (16, 17). There are currently two main cryopreservation methods, respectively conventional slow freezing and vitrification(18, 19). Before cryopreservation, the ovarian tissue should be obtained by laparoscopy or laparotomy. The ovarian tissue with immature germ cells of one or both ovaries is separated into small pieces about 5 × 5 mm2 with a thickness of 1-3 mm before cryopreservation(20).
Conventional slow freezing uses a controlled-rate freezer to gradually cool ovarian tissue with a lower concentration of cryoprotectant, minimizing ice crystal formation and reducing cell damage. Cryoprotectants help limit intracellular ice but may be cytotoxic. Extending freezing time and lowering cryoprotectant levels further reduce cell damage risks. After freezing, the tissue is stored in cryovials within liquid nitrogen at around -196°C.(21, 22).

Vitrification rapidly cools ovarian tissue to minimize intracellular ice formation, using higher concentrations of cryoprotectants like Dimethyl sulfoxide (DMSO), glycerol, propylene glycol (PrOH), or ethylene glycol (EG) as osmotic agents. While high concentrations increase cytotoxicity, they reduce tissue stress by limiting ice formation. Minimizing exposure to these agents also lowers cell damage risk. Rapid cooling turns the solution into a glass-like, amorphous state, and the tissue is stored in liquid nitrogen at approximately -196°C.(23-25).

Thawing is a crucial step in cryopreservation, warming frozen tissue back to physiological temperature before transplantation. The ovarian tissue is gradually warmed to prevent intracellular ice crystal formation, using specialized warming devices. Afterward, cryoprotectants are removed, and histological examination assesses tissue quality.(26).

Histological examination of ovarian tissue is essential for evaluating the success of ovarian tissue cryopreservation and forms the basis for ovarian transplantation. Follicle viability is the most important aspect in the histological examination of ovarian tissue, serving as a key indicator of fertility (27, 28). Common methods are hematoxylin-eosin (HE) staining, dead and alive cell detection, etc. Since freezing can increase DNA damage, assessing DNA integrity is also essential and is a critical criterion for evaluating the impact of ovarian tissue cryopreservation (29, 30). The stromal cells of the ovary are a key component of the ovarian tissue, playing a crucial role in supporting the development and function of ovarian follicles(31). Therefore, the viability and number of stromal cells in the ovarian tissue cryopreservation also determine the growth of follicles and ovarian function after thawing(32). After thawing the ovarian tissue, ovarian transplantation is the best way to preserve the patient's fertility and endocrine function. In addition, there are several other ways to culture immature follicles and then transplant them. The culture methods are in vitro follicle culture, in vitro maturation of immature oocytes, and artificial ovary(33, 34). The schematic overview of ovarian cryopreservation is shown in Fig.1.

This article aimed to perform a systematic review and meta-analysis to assess the condition of primary and secondary oocytes, stromal cells, and DNA damage in ovarian tissues following conventional slow freezing and vitrification, providing a quality evaluation.

3.3. Methods

Search strategy

The study was conducted according to the meta-analysis of observational studies in epidemiology (MOOSE) guidelines. We performed a literature search using the keywords “ovarian tissue”, “ovary”, follicle”, “cryopreservation”, and “vitrification” in various combinations to January 2024 in PubMed, Embase, and Cochrane Library. We conducted a Pubmed search using the following search string: ("ovary"[MeSH Terms] OR "ovarial"[All Fields] OR "ovary"[All Fields] OR "ovaries"[All Fields] OR "ovary s"[All Fields] OR (("ovarian"[All Fields] OR "ovarians"[All Fields]) AND ("tissue s"[All Fields] OR "tissues"[MeSH Terms] OR "tissues"[All Fields] OR "tissue"[All Fields])) OR ("follicle s"[All Fields] OR "hair follicle"[MeSH Terms] OR ("hair"[All Fields] AND "follicle"[All Fields]) OR "hair follicle"[All Fields] OR "follicles"[All Fields] OR "ovarian follicle"[MeSH Terms] OR ("ovarian"[All Fields] AND "follicle"[All Fields]) OR "ovarian follicle"[All Fields] OR "follicle"[All Fields])) AND ("cryopreservability"[All Fields] OR "cryopreservable"[All Fields] OR "cryopreservant"[All Fields] OR "cryopreservants"[All Fields] OR "cryopreservated"[All Fields] OR "cryopreservation"[MeSH Terms] OR "cryopreservation"[All Fields] OR "cryopreserved"[All Fields] OR "cryopreservations"[All Fields]

OR "cryopreservative"[All Fields] OR "cryopreservatives"[All Fields] OR "cryopreserve"[All Fields] OR "cryopreserving"[All Fields]) AND ("vitrificated"[All Fields] OR "vitrification"[MeSH Terms] OR "vitrification"[All Fields]). We conducted an Embase search using the following search string: (('ovary'/exp OR ovary OR ovarian) AND ('tissue'/exp OR tissue) OR 'follicle'/exp OR follicle) AND ('cryopreservation'/exp OR cryopreservation) AND ('vitrification'/exp OR vitrification). We conducted a Cochrane Library search using the following search string: (ovary OR ovarian tissue OR follicle) AND cryopreservation AND vitrification. The search All studies were assessed by two investigators independently and any difference was settled by discussion. Studies in all languages were included.

Study selection criteria and exclusion criteria

Research articles comparing the follicular viability of conventional slow cryopreservation and vitrification were considered appropriate for the analysis. Studies that were not classified as research articles were excluded. And studies focused on other topics or with insufficient data for follicular viability were also excluded.

Outcome measures

Primary outcomes

- Follicular viability (the proportion of viable follicles in the total follicles)
- The proportion of intact primordial follicles (the proportion of intact primordial follicles in the total primordial follicles)

Secondary outcomes

- The proportion of DNA fragmented follicles (the proportion of DNA damage follicles in the total follicles)
- The proportion of stromal cells (the proportion of intact stromal cells in the total stromal cells)

Data abstraction and quality assessment

The following data were extracted: baseline characteristics including age range, mean age, how patients were found, the reason for exclusion of participants, surgical techniques, the method of follicles quality evaluation, the freezing solution of conventional slow freezing and vitrification, follicular density, follicular viability, the proportion of intact primordial follicles, the proportion of DNA fragmented follicles. The risk of bias in individual studies was assessed by the Funnel plot bias. The Newcastle-Ottawa Scale for evidence-based medicine checklist about each risk of bias item as percentages across all included studies were used to evaluate the methodological quality.

Statistical analysis

This meta-analysis was performed using Stata software (Version 12.0) and Review Manager (Version 5.2). We used the Cochran Q test to evaluate the heterogeneity. Heterogeneity was used to evaluate the percentage of the variation in all studies. Because of inevitable clinical and methodologic diversity, inconsistency (I^2) was adopted to quantify the effect of statistical heterogeneity. A value of 0% indicated no observed heterogeneity and a value of more than 50% was considered substantial heterogeneity. If $I^2 > 50\%$, the random-effects model was adopted, otherwise, the fixed-effects model was used to pool the results. Review Manager (Version 5.2) was used to do the risk of bias graph. A two-sided $P < 0.05$ was considered statistically significant.

3.4. Results

Study characteristics

The search process is shown in Fig. 2. The number of records identified through PubMed, Embase is 1353. The total number of records is 1394 added the records identified through the Cochrane Library. After screening titles and abstracts, 32 publications remained. In the 32 publications, 5 of them have no comparison group, 2 of them are the analysis of xenotransplantation, and 7 of them have no sufficient data. Finally, 18 publications were selected for this meta-analysis(35-52).

Details of the 18 publications are shown in Table 1 and Table 2. All the included studies are published between 2007 and 2022. Published at most in 2022 is 3 articles. The included studies are from 10 countries, China for 5 studies is the most, and Germany for 3 is the second. The mean age range is nearly 20-40 years old. Laparoscopy surgery is the most common surgical approach 7 publications use laparoscopy surgery to obtain ovarian tissue, and 4 publications use laparoscopy surgery or open surgery to obtain ovarian tissue. All cryopreservation ovarian tissue was analyzed by light microscopy.

Primary outcome

Follicular viability

The follicular viability of conventional slow cryopreservation versus vitrification in the 18 publications was analyzed. The pooled results indicated that there was no difference in follicular viability between the two approaches from random effect analysis (RR = 0.96, 95% CI: 0.84–1.09, I2 = 95.8%, P< 0.001 for heterogeneity, P = 0.520 shown in Fig. 3A). S.Silber et al.'s study reported vitrification was associated with significantly higher follicular viability

(RR=0.47, 95% CI: 0.43–0.52)(40). Other two studies from A. Dalman et al. (RR=1.07, 95% CI: 1.01–1.12) and S.Zhao et al. (RR=1.16, 95% CI: 1.08–1.25) reported conventional slow cryopreservation was associated with higher follicular viability(42, 49). Other studies reported there was no difference in follicular viability between the two approaches.

The proportion of intact primordial follicles

The proportion of intact primordial follicles of conventional slow cryopreservation versus vitrification in the 8 publications was analyzed. The other 10 publications did not report the data of the proportion of intact primordial follicles. The pooled results indicated that there was no difference in the proportion of intact primordial follicles between the two approaches from random effect analysis (RR=1.01, 95% CI: 0.94–1.09, I2=70.6%, P= 0.001 for heterogeneity, P=0.778 shown in Fig. 3B). S.Zhao et al.'s study reported conventional slow cryopreservation was associated with higher intact primordial follicles (RR=1.18, 95% CI: 1.09–1.26)(42). Other studies reported there was no difference in intact primordial follicles between the two approaches.

Second outcome

The proportion of DNA fragmented follicles

The proportion of DNA fragmented follicles of conventional slow cryopreservation versus vitrification in the 5 publications was analyzed. The other 13 publications did not report the data of the proportion of DNA fragmented follicles. The pooled results indicated that there was no difference in the proportion of DNA fragmented follicles between the two approaches (RR=1.20, 95% CI: 0.94–1.54, I2=0.0%, P= 0.675 for heterogeneity, P=0.151 shown in Fig. 4A).

The proportion of stromal cells

The proportion of stromal cells of conventional slow cryopreservation versus vitrification in the 3 publications was analyzed. The other 15 publications did not report the data of the proportion of stromal cells. The pooled results indicated that there was no difference in the proportion of stromal cells between the two approaches from random effect analysis (RR=0.58, 95% CI: 0.20–1.65, I2=99.7%, P<0.001 for heterogeneity, P=0.303 shown in Fig. 4B). The study from V. Keros et al. (RR=0.23, 95% CI: 0.22–0.23) and H. J. Chang et al. (RR=0.79, 95% CI: 0.72–0.86) reported vitrification was associated with significantly higher stromal cells(48, 52). The other study reported there was no difference in the proportion of stromal cells between the two approaches.

Risk of bias in included studies

Funnel plot bias of the studies included in this meta-analysis was notably symmetrical indicating that there was no obvious bias in publication shown in Fig. 5. According to the Newcastle-Ottawa Scale for evidence-based medicine checklist about each risk of bias item as percentages across all included studies, the risk of bias graph was shown in Fig. 6.

3.5. Discussion

The meta-analysis is to compare the quality of ovarian tissue cryopreservation by conventional slow cryopreservation and vitrification. A total of 18 articles met the criteria were included. No more articles were included due to that ovarian tissue cryopreservation is not a routine method for every reproductive center, more centers use oocyte cryopreservation to preserve patients' fertility(53, 54). First, due to complex ethical methods, ovarian tissue cryopreservation has not yet become a routine method recognized by the public. Second, oocyte cryopreservation can meet the basic requirements of patients for preserving fertility. The cryopreservation of ovarian tissue aims to better preserve endocrine function, allowing follicles to mature and develop more effectively, thereby more accurately maintaining the patient's fertility (55-58). In recent years, with advancements in science and technology and increased awareness, the number of ovarian tissue cryopreservation has increased gradually(27, 52, 59, 60). There is also controversy about the method of ovarian tissue cryopreservation(61). Conventional slow cryopreservation can minimize the formation of intracellular ice crystals during the freezing process through gradient cooling. To ensure cell viability, this method has been widely used since the development of ovarian cryopreservation and has achieved good results. The first ovarian transplant birth was the application of conventional slow cryopreservation(62). However, conventional slow cryopreservation takes a long time and leads to cells death, especially increased(63). Recently, vitrification has been gradually popular. The vitrification can freeze tissues more quickly though speeding up the freezing by increasing the concentration of cryoprotectant. However, vitrification will lead to the loss of cytoskeletal elements and is more likely to lead to cellular ischemia (64, 65). As whether the newer freezing method can replace the conventional slow cryopreservation method has become a topic of significant consideration.

3.5.1. The primary outcomes

3.5.1.1. Follicular viability

As we all know, in ovarian cryopreservation, the most important evaluation criterion is follicular viability(28, 66, 67). Follicular viability is the basis for determining the patient's subsequent fertility and is also an important indicator for evaluating ovarian cryopreservation methods.

Comparing follicular viability between the two cryopreservation methods, we find that S. Silber et al.'s study reported significantly higher follicular viability with vitrification, with viability rates of 41.7% for slow freeze-cryopreserved tissue and 89.1% for vitrified tissue(40). This is quite different from other studies. Without subjective bias, we analyze the reasons for the difference of follicular viability in conventional slow cryopreservation and vitrification. First, in this article, the freezing solution of conventional slow cryopreservation did not contain human serum albumin (HSA) a cryoprotectant that helps protect cells by reducing cellular ice crystal formation(68). The article was published in 2010, and followed the ovarian transplants for up to 5 years, so the method may be used in 2005 or before. The HSA may not be used in those years as cryoprotectant. Recently, HSA has been widely used for ovarian tissue cryopreservation. In addition to reducing the formation of intracellular ice crystals, it can also maintain cell osmotic pressure and enhance cellular antioxidant capacity(69). Although it is not an essential component of cryoprotectants, with it can reduce cell damage. Second, the follicles quality evaluation method of this article is different from other articles. Most other articles use microscope counting to judge. This article uses propidium iodide (PI) staining and flow cytometry for detection and assessment. Microscopic counting may not effectively observe subtle changes in cell apoptosis, as early-stage apoptotic cells are harder to identify. However, PI staining can accurately identify cell status. Additionally, transmission electron microscopy is used to observe the cells in this study. The result is that most of the stroma cells in the slow freeze–cryopreserved specimen was lysed and their nuclei compressed between dense bundles of extracellular fibers. Stroma cells are very important in the follicle maturation process, so it may be that the damage to stromal cells caused by conventional slow cryopreservation further led to the damage to follicle cells (70, 71).Third, the freezing cryoprotectant of vitrification is 7.5% ethylene glycol (EG) and 7.5% DMSO for 25 mins followed by a second equilibration in 20% EG and 20% DMSO for 15 mins. The first concentration is lower than other vitrification methods. Additionally, synthetic serum substitute (SSS) was added as a protective agent, which may contribute to better follicular viability. However, A. Dalman et al. and S.Zhao et al. reported conventional slow cryopreservation was associated with higher follicular viability. A. Dalman et al. reported the follicular viability of conventional slow cryopreservation was 97.4 % and vitrification was 91.4%(49). The follicular viability in this study is higher than in other studies. This study uses microscope counting for detection and assessment. There may be some subjective bias. At the same time, vitrification was correlated with more free radicals, and reactive oxygen species (ROS) (72). Oxidative stress occurs when more ROS produced, which will result in damage to cellular components such as DNA, proteins, and lipids and cause cell damage(73, 74). Adding catalase to the cryoprotectant may reduce the production of ROS and protect freezing cells(75). S.Zhao et al. reported the follicular viability of conventional slow cryopreservation was 82.9 % and

vitrification was 71.3%(42). The main reason for this difference is that this study used large pieces of human ovarian tissues that is 15 mm × 15 mm × 2mm which is larger than other studies. The larger ovarian tissue cryopreservation may reduce follicles loss and support follicles growth(76, 77). The higher density of the vitrification cryoprotectant prevents it from quickly penetrating large tissues, resulting in uneven vitrification of the cellular water throughout the tissue. This may be the reason for conventional slow cryopreservation was associated with higher follicular viability.

3.5.1.2. The proportion of intact primordial follicles

Primordial follicles are the earliest stage of ovarian follicles, which account for more than 90% of the population of follicles(78, 79). During ovarian tissue cryopreservation, a higher proportion of intact primordial follicles indicates greater fertility potential(43, 80). Comparing the proportion of intact primordial follicles between the two cryopreservation methods, S.Zhao et al. reported the proportion of intact primordial follicles of conventional slow cryopreservation was 86.8 % and vitrification was 73.8%(42). As previously mentioned, this study used large pieces of human ovarian tissues, which may lead to differing results between the two methods. Larger tissue samples may benefit from conventional slow cryopreservation not vitrification, which may not penetrate large tissues quickly enough, potentially leading to the formation of intracellular ice crystals that can damage cells. More studies about this supposed need to be verified. Additionally, while the morphology of frozen primordial follicles may appear normal, their subsequent development, fertilization, and the ability to achieve pregnancy and live birth can be influenced by various factors(54, 81). Therefore, fertility outcomes cannot be solely determined by normal morphology; it represents just the initial step in assessing the efficiency of cryopreservation.

3.5.2. Secondary outcomes

3.5.2.1. The proportion of DNA fragmented follicles

According to this meta-analysis, there was no difference in the proportion of DNA fragmented follicles between the two approaches. Both methods can lead to cell apoptosis, mainly manifested as DNA damage. This is mainly attributed to the inevitable formation of intracellular ice crystals during the freezing process, despite efforts by cryoprotectants to minimize their formation. The formation of intracellular ice crystals can cause mechanical damage, which can further lead to rupture of cell membranes and other cellular structures. Moreover, it can cause structural damage by causing damage and breakage of DNA strands due to reduced space for cell expansion(82, 83). In addition, cryoprotectants containing that may cause chemical

damage and directly damage DNA(84). Therefore, reducing DNA damage during the freezing process should also be considered in future freezing methods.

3.5.2.2. The proportion of stromal cells

Stromal cells are an essential part of ovarian tissue providing structural support for follicles and contributing to the production of extracellular matrix components and hormones. They provide the necessary microenvironment for the growth and development of oocytes and play an important role in the development and maturation of follicles(85, 86). Research shows that the weakened antioxidant capacity of stromal cells is closely related to ovarian aging(87). More attention should be paid to the survival status of stromal cells in ovarian tissue cryopreservation. The study of H. J. Chang et al. showed that the proportion of stromal cells of conventional slow cryopreservation was 47.8% and vitrification was 60.7%. Vitrification may reduce damage to stromal cells, possibly because stromal cells are relatively small. During vitrification, they can quickly reach the vitrified state, reducing the formation of intracellular ice crystals, and thereby reducing cell damage(69). However, it's important to note that the vitrification method is not standardized, and further studies using consistent vitrification protocols are needed for comprehensive evaluation.

3.5.3. Limitations

This meta-analysis reviewed the comparison of ovarian tissue cryopreservation by conventional slow cryopreservation and vitrification till January 2024. Our results indicate comparable outcomes for ovarian tissue cryopreservation between the two approaches. The main limitation of our study is the significant variation in the methods of conventional slow cryopreservation and vitrification. This variability arises from differences in the composition and ratio of cryoprotectants used. They mainly contain such as EG, DMSO, PrOH, etc. The difference between the different cryoprotectants is not particularly obvious. There is currently no unified regulation. Studies by Meryman indicated that osmotic effects may be the major factors in slow-freezing cell injury(88). The same group demonstrated that adding neutral solutes as cryoprotective agents (CPAs) can reduce osmotically driven cell injury during ice solidification due to colligative effects(89). There are two main categories of cryoprotective agents(90, 91). The first is penetrating cryoprotective agents. DMSO is one of the most effective and widely used cryoprotective agents which is a small, amphipathic molecule that can easily penetrate cell membranes and distribute throughout both intracellular and extracellular environments(92). During the freezing process, DMSO creates an osmotic gradient that helps dehydrate cells, reducing the amount of free water inside them and thereby

decreasing the chances of intracellular ice formation. By controlling the osmotic balance, it also helps prevent osmotic shock during freezing and thawing, which can lead to cell lysis or severe deformation. The concentration of DMSO used in cryopreservation typically ranges from 5% to 10%(93-95). EG is another effective cryoprotective agent that has a similar function to DMSO. The main difference is that EG is a small, water-soluble molecule. The concentration of ethylene glycol used in cryopreservation protocols typically ranges from 10% to 20%(96, 97). In many studies, a mixture of DMSO and EG is reported to be more effective as the cryoprotectant(93, 98-100). The other cryoprotective agents are non-penetrating cryoprotective agents with sucrose being the primary one. The combination of different cryoprotectants aims to enhance osmotic pressure regulation inside and outside cells, prevent intracellular ice crystal formation, and stabilize cell membranes and proteins. Different researchers utilize varying combinations of cryoprotectants based on their individual research experiences(93, 94, 101, 102). Moreover, follicular viability, the proportion of intact primordial follicles, the proportion of DNA fragmented in follicles, and the proportion of stromal cells are essential criteria for evaluating ovarian tissue cryopreservation. The clinical pregnancy rate is also very important in ovarian tissue cryopreservation and is the most crucial metric for evaluating female reproductive function. However, under current clinical conditions, experience is relatively limited. Beyond evaluating ovarian cryopreservation technology, the success of ovarian tissue cryopreservation also depends on transplantation surgery techniques, the method of conception, and the physical condition of the female patient, including factors such as pelvic radiation and prior chemotherapy. And until now, the ovarian tissue cryopreservation is considered an experimental procedure. More studies are needed.

3.6. Conclusions

In conclusion, this meta-analysis indicated that conventional slow cryopreservation and vitrification appear to provide comparable outcomes of follicular viability, the proportion of intact primordial follicles, the proportion of DNA fragmented follicles and the proportion of stromal cells. However, included studies varied in the use of cryopreservation protocols. Therefore, further studies are needed to determine the optimal method for cryopreservation human ovarian tissue. While there is no significant difference between traditional cryopreservation and vitrification regarding ovarian tissue anatomy, each method has its own advantages and is suitable for different clinical scenarios. The advantages of traditional cryopreservation include its mature technology and relatively standardized procedures. On the other hand, vitrification offers benefits such as fast freezing speed, simplicity of operation, and high flexibility. And until now, the ovarian tissue cryopreservation is considered an experimental procedure. The choice of method can be tailored to meet specific clinical needs.

Abbreviations

DMSO: Dimethyl sulfoxide, PrOH: glycerol, propylene glycol, EG: ethylene glycol, HAS: human serum albumin, SSS: synthetic serum substitute, ROS: reactive oxygen species.

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Authors' contributions

QDK contributed to the design of the meta-analysis, data analysis, and the completion of the article. CP, GR, and PR contributed to the document selection and the inclusion and exclusion criteria. VI contributed to the design and review of the meta-analysis. All authors approved the final manuscript.

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Availability of data and materials

All data is available in this paper.

Declarations:

Ethics approval and consent to participate
This study does not contain any studies with human participants or animals performed.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1. Department of Obstetrics and Gynecology, Medical Faculty, Cologne University, 50931 Cologne, Germany.
2. Medizinisches Versorgungszentrum AMEDES für IVF- und Pränatalmedizin in Köln GmbH, 50968 Cologne, Germany.

3.7. References

1. Dolmans MM, Donnez J, Cacciottola L. Fertility Preservation: The Challenge of Freezing and Transplanting Ovarian Tissue. Trends Mol Med. 2021;27(8):777-91.
2. Donnez J, Dolmans MM. Fertility preservation in men and women: Where are we in 2021? Are we rising to the challenge? Fertil Steril. 2021;115(5):1089-90.
3. Yding Andersen C, Mamsen LS, Kristensen SG. FERTILITY PRESERVATION: Freezing of ovarian tissue and clinical opportunities. Reproduction. 2019;158(5):F27-f34.
4. Schallmoser A, Einenkel R, Färber C, Hüren V, Pougin A, Emrich N, et al. Cryostorage of human ovarian tissue: evaluating the storage and disposal pattern over a 22-year period in 2475 patients. Reprod Biomed Online. 2023;47(3):103239.
5. Duffin K, Howie R, Kelsey TW, Wallace HB, Anderson RA. Long-term follow-up to assess criteria for ovarian tissue cryopreservation for fertility preservation in young women and girls with cancer. Hum Reprod. 2023;38(6):1076-85.
6. Dolmans MM, Donnez J. Fertility preservation in women for medical and social reasons: Oocytes vs ovarian tissue. Best practice & research Clinical obstetrics & gynaecology. 2021;70:63-80.
7. Ruan X, Cheng J, Du J, Jin F, Gu M, Li Y, et al. Analysis of Fertility Preservation by Ovarian Tissue Cryopreservation in Pediatric Children in China. Front Endocrinol (Lausanne). 2022;13:930786.
8. Rives N, Courbière B, Almont T, Kassab D, Berger C, Grynberg M, et al. What should be done in terms of fertility preservation for patients with cancer? The French 2021 guidelines. Eur J Cancer. 2022;173:146-66.
9. Fabbri R, Vicenti R, Magnani V, Paradisi R, Lima M, De Meis L, et al. Ovarian tissue cryopreservation and transplantation: 20 years experience in Bologna University. Front Endocrinol (Lausanne). 2022;13:1035109.
10. Newton H, Fisher J, Arnold JR, Pegg DE, Faddy MJ, Gosden RG. Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. Hum Reprod. 1998;13(2):376-80.

11. Khattak H, Malhas R, Craciunas L, Afifi Y, Amorim CA, Fishel S, et al. Fresh and cryopreserved ovarian tissue transplantation for preserving reproductive and endocrine function: a systematic review and individual patient data meta-analysis. Hum Reprod Update. 2022;28(3):400-16.
12. Blumenfeld Z. Ovarian tissue transplantation and leukemia. Fertil Steril. 2018;109(1):69-70.
13. Silber SJ, DeRosa M, Goldsmith S, Fan Y, Castleman L, Melnick J. Cryopreservation and transplantation of ovarian tissue: results from one center in the USA. Journal of assisted reproduction and genetics. 2018;35(12):2205-13.
14. Isachenko V, Morgenstern B, Todorov P, Isachenko E, Mallmann P, Hanstein B, et al. Patient with ovarian insufficiency: baby born after anticancer therapy and re-transplantation of cryopreserved ovarian tissue. Journal of ovarian research. 2020;13(1):118.
15. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. N Engl J Med. 2000;342(25):1919.
16. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet. 2004;364(9443):1405-10.
17. Dolmans MM, von Wolff M, Poirot C, Diaz-Garcia C, Cacciottola L, Boissel N, et al. Transplantation of cryopreserved ovarian tissue in a series of 285 women: a review of five leading European centers. Fertil Steril. 2021;115(5):1102-15.
18. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update. 2017;23(2):139-55.
19. Jaeger P, Fournier C, Santamaria C, Fraison E, Morel-Journel N, Benchaib M, et al. Human ovarian cryopreservation: vitrification versus slow freezing from histology to gene expression. Hum Fertil (Camb). 2023;26(5):1099-107.
20. Meirow D, Baum M, Yaron R, Levron J, Hardan I, Schiff E, et al. Ovarian tissue cryopreservation in hematologic malignancy: ten years' experience. Leuk Lymphoma. 2007;48(8):1569-76.
21. Isachenko V, Isachenko E, Reinsberg J, Montag M, Braun F, van der Ven H. Cryopreservation of human ovarian tissue: effect of spontaneous and initiated ice formation. Reprod Biomed Online. 2008;16(3):336-45.
22. Sanfilippo S, Canis M, Romero S, Sion B, Déchelotte P, Pouly JL, et al. Quality and functionality of human ovarian tissue after cryopreservation using an original slow freezing procedure. J Assist Reprod Genet. 2013;30(1):25-34.

23. Isachenko V, Montag M, Isachenko E, van der Ven K, Dorn C, Roesing B, et al. Effective method for in-vitro culture of cryopreserved human ovarian tissue. Reprod Biomed Online. 2006;13(2):228-34.
24. Isachenko E, Isachenko V, Nawroth F, Rahimi G, Kreienberg R, Reinsberg J, et al. Human ovarian tissue preservation: is vitrification acceptable method for assisted reproduction? Cryo letters. 2008;29(4):301-14.
25. Martínez-Burgos M, Herrero L, Megías D, Salvanes R, Montoya MC, Cobo AC, et al. Vitrification versus slow freezing of oocytes: effects on morphologic appearance, meiotic spindle configuration, and DNA damage. Fertil Steril. 2011;95(1):374-7.
26. Isachenko V, Dittrich R, Keck G, Isachenko E, Rahimi G, van der Ven H, et al. Cryopreservation of Ovarian Tissue: Detailed Description of Methods for Transport, Freezing and Thawing. Geburtshilfe Frauenheilkd. 2012;72(10):927-32.
27. Isachenko V, Orth I, Isachenko E, Mallmann P, Peters D, Schmidt T, et al. Viability of human ovarian tissue confirmed 5 years after freezing with spontaneous ice-formation by autografting and chorio-allantoic membrane culture. Cryobiology. 2013;66(3):233-8.
28. Isachenko V, Isachenko E, Kreienberg R, Woriedh M, Weiss J. Human ovarian tissue cryopreservation: quality of follicles as a criteria of effectiveness. Reproductive biomedicine online. 2010;20(4):441-2.
29. Kim B, Ryu KJ, Lee S, Kim T. Changes in telomere length and senescence markers during human ovarian tissue cryopreservation. Sci Rep. 2021;11(1):2238.
30. Noda M, Ma Y, Yoshikawa Y, Imanaka T, Mori T, Furuta M, et al. A single-molecule assessment of the protective effect of DMSO against DNA double-strand breaks induced by photo-and γ-ray-irradiation, and freezing. Sci Rep. 2017;7(1):8557.
31. Shen L, Liu J, Luo A, Wang S. The stromal microenvironment and ovarian aging: mechanisms and therapeutic opportunities. J Ovarian Res. 2023;16(1):237.
32. Xia X, Yin T, Yan J, Yan L, Jin C, Lu C, et al. Mesenchymal Stem Cells Enhance Angiogenesis and Follicle Survival in Human Cryopreserved Ovarian Cortex Transplantation. Cell Transplant. 2015;24(10):1999-2010.
33. Cho E, Kim YY, Noh K, Ku SY. A new possibility in fertility preservation: The artificial ovary. J Tissue Eng Regen Med. 2019;13(8):1294-315.
34. Telfer EE, Fauser BC. Important steps towards materializing the dream of developing an artificial ovary. Reprod Biomed Online. 2016;33(3):333-4.
35. Borrás A, Manau D, Fabregues F, Peralta S, Calafell JM, Casals G, et al. Comparison between slow freezing and vitrification of ovarian tissue cryopreservation in assigned female at birth transgender people receiving testosterone therapy: data on histological and viability parameters. Journal of assisted reproduction and genetics. 2022;39(2):527-41.

36. Galbinski S, Kowalewski LS, Grigolo GB, da Silva LR, Jiménez MF, Krause M, et al. Comparison between two cryopreservation techniques of human ovarian cortex: morphological aspects and the heat shock response (HSR). Cell Stress and Chaperones. 2021;27(2):97-106.
37. Sugishita Y, Taylan E, Kawahara T, Shahmurzada B, Suzuki N, Oktay K. Comparison of open and a novel closed vitrification system with slow freezing for human ovarian tissue cryopreservation. Reproductive sciences (Thousand Oaks, Calif). 2021;38(10):2723-33.
38. Huang L, Mo Y, Wang W, Li Y, Zhang Q, Yang D. Cryopreservation of human ovarian tissue by solid-surface vitrification. European journal of obstetrics, gynecology, and reproductive biology. 2008;139(2):193-8.
39. Nikhil Kumar Tej J, Somoskoi B, Cseh S, Ramos L, Galbinski S, Nacul A, et al. Detailed Morphological Analysis of Cryoinjury in Human Ovarian Tissue Following Vitrification or Slow Freezing. Reproduction in domestic animals = Zuchthygiene. 2022;29(8):2374-81.
40. Silber S, Kagawa N, Kuwayama M, Gosden R. Duration of fertility after fresh and frozen ovary transplantation. Fertility and sterility. 2010;94(6):2191-6.
41. Schallmoser A, Einenkel R, Färber C, Emrich N, John J, Sänger N. The effect of high-throughput vitrification of human ovarian cortex tissue on follicular viability: a promising alternative to conventional slow freezing? Archives of gynecology and obstetrics. 2023;307(2):591-9.
42. Zhao S, Zhao SQ, Shi Q, Qin XM, Han YL, Chao L, et al. Effects of different freezing methods on the follicular viability of large pieces of human ovarian tissues. Acta Anatomica Sinica. 2013;44(3):427-32.
43. Isachenko V, Lapidus I, Isachenko E, Krivokharchenko A, Kreienberg R, Woriedh M, et al. Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation. Reproduction (Cambridge, England). 2009;138(2):319-27.
44. Wang TR, Yan J, Lu CL, Xia X, Yin TL, Zhi X, et al. Human single follicle growth in vitro from cryopreserved ovarian tissue after slow freezing or vitrification. Human reproduction (Oxford, England). 2016;31(4):763-73.
45. Li YX, Sun YP, Tang L, Ma YP. Influence of different cryopreservation schemes on human ovarian tissue activity. Chinese Journal of Tissue Engineering Research. 2013;17(53):9125-31.
46. Li YB, Zhou CQ, Yang GF, Wang Q, Dong Y. Modified vitrification method for cryopreservation of human ovarian tissues. Chinese medical journal. 2007;120(2):110-4.
47. Fabbri R, Vicenti R, Macciocca M, Martino NA, Dell'Aquila ME, Pasquinelli G, et al. Morphological, ultrastructural and functional imaging of frozen/thawed and vitrified/warmed human ovarian tissue retrieved from oncological patients. Human reproduction (Oxford, England). 2016;31(8):1838-49.

48. Chang HJ, Moon JH, Lee JR, Jee BC, Suh CS, Kim SH. Optimal condition of vitrification method for cryopreservation of human ovarian cortical tissues. The journal of obstetrics and gynaecology research. 2011;37(8):1092-101.
49. Dalman A, Deheshkar Gooneh Farahani NS, Totonchi M, Pirjani R, Ebrahimi B, Rezazadeh Valojerdi M. Slow freezing versus vitrification technique for human ovarian tissue cryopreservation: An evaluation of histological changes, WNT signaling pathway and apoptotic genes expression. Cell and tissue banking. 2017;79:29-36.
50. Klocke S, Bündgen N, Köster F, Eichenlaub-Ritter U, Griesinger G. Slow-freezing versus vitrification for human ovarian tissue cryopreservation. Archives of gynecology and obstetrics. 2015;291(2):419-26.
51. Sanfilippo S, Canis M, Dechelotte P, Artonne C, Janny L, Brugnon F. Vitrification versus slow freezing: What is the better method to preserve human ovarian tissue quality? Fertility and sterility. 2012;98(3):S130-S1.
52. Keros V, Xella S, Hultenby K, Pettersson K, Sheikhi M, Volpe A, et al. Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue. Human reproduction (Oxford, England). 2009;24(7):1670-83.
53. Hossay C, Tramacere F, Cacciottola L, Camboni A, Squifflet JL, Donnez J, et al. Follicle outcomes in human ovarian tissue: effect of freezing, culture, and grafting. Fertility and sterility. 2023;119(1):135-45.
54. Fabbri R. Cryopreservation of human oocytes and ovarian tissue. Cell and tissue banking. 2006;7(2):113-22.
55. Bromer JG, Patrizio P. Fertility preservation: the rationale for cryopreservation of the whole ovary. Seminars in reproductive medicine. 2009;27(6):465-71.
56. Silber SJ. Ovary cryopreservation and transplantation for fertility preservation. Molecular human reproduction. 2012;18(2):59-67.
57. Amorim CA, Dolmans MM, David A, Jaeger J, Vanacker J, Camboni A, et al. Vitrification and xenografting of human ovarian tissue. Fertility and sterility. 2012;98(5):1291-8.e1-2.
58. Ovarian tissue cryopreservation: a committee opinion. Fertility and sterility. 2014;101(5):1237-43.
59. Rosendahl M, Schmidt KT, Ernst E, Rasmussen PE, Loft A, Byskov AG, et al. Cryopreservation of ovarian tissue for a decade in Denmark: a view of the technique. Reprod Biomed Online. 2011;22(2):162-71.
60. Bastings L, Liebenthron J, Westphal JR, Beerendonk CC, van der Ven H, Meinecke B, et al. Efficacy of ovarian tissue cryopreservation in a major European center. J Assist Reprod Genet. 2014;31(8):1003-12.
61. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. Human reproduction update. 2012;18(5):536-54.

62. Maffei S, Hanenberg M, Pennarossa G, Silva JR, Brevini TA, Arav A, et al. Direct comparative analysis of conventional and directional freezing for the cryopreservation of whole ovaries. Fertility and sterility. 2013;100(4):1122-31.
63. Zhang JM, Wang HX, Lu XL. Damage of Granusola and Stroma Cells Exposed to Mouse Ovarian Tissue Cryopreservation: Potential Mechanism of Ovarian Injury. Cryo letters. 2021;42(1):53-8.
64. Wang D, Geng M, Gan D, Han G, Gao G, Xing A, et al. Effect of resveratrol on mouse ovarian vitrification and transplantation. Reproductive biology and endocrinology : RB&E. 2021;19(1):54.
65. Lornage J, Salle B. Ovarian and oocyte cryopreservation. Current opinion in obstetrics & gynecology. 2007;19(4):390-4.
66. Marschalek J, Hager M, Wanderer S, Ott J, Frank M, Schneeberger C, et al. Different Impacts of Cryopreservation in Endothelial and Epithelial Ovarian Cells. International journal of molecular sciences. 2023;24(15).
67. Behl S, Joshi VB, Larson NB, Young MC, Bilal M, Walker DL, et al. Vitrification versus slow freezing of human ovarian tissue: a systematic review and meta-analysis of histological outcomes. Journal of assisted reproduction and genetics. 2023;40(3):455-64.
68. Hreinsson J, Zhang P, Swahn ML, Hultenby K, Hovatta O. Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. Human reproduction (Oxford, England). 2003;18(11):2420-8.
69. Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab. 2002;87(1):316-21.
70. Kinnear HM, Tomaszewski CE, Chang FL, Moravek MB, Xu M, Padmanabhan V, et al. The ovarian stroma as a new frontier. Reproduction (Cambridge, England). 2020;160(3):R25-r39.
71. Fienberg R, Cohen RB. A COMPARATIVE HISTOCHEMICAL STUDY OF THE OVARIAN STROMAL LIPID BAND, STROMAL THECA CELL, AND NORMAL OVARIAN FOLLICULAR APPARATUS. American journal of obstetrics and gynecology. 1965;92:958-69.
72. Cheng J, Ruan X, Zhou Q, Li Y, Du J, Jin F, et al. Long-time low-temperature transportation of human ovarian tissue before cryopreservation. Reproductive biomedicine online. 2021;43(2):172-83.
73. Cacciottola L, Manavella DD, Amorim CA, Donnez J, Dolmans MM. In vivo characterization of metabolic activity and oxidative stress in grafted human ovarian tissue using microdialysis. Fertility and sterility. 2018;110(3):534-44.e3.

74. Rahimi G, Isachenko E, Sauer H, Isachenko V, Wartenberg M, Hescheler J, et al. Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. Reproduction, fertility, and development. 2003;15(6):343-9.
75. Tavakoli A, Aliakbari F, Soleimani Mehranjani M. Kisspeptin decreases the adverse effects of human ovarian vitrification by regulating ROS-related apoptotic occurrences. Zygote (Cambridge, England). 2023;31(6):537-43.
76. Aubard Y, Piver P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. Hum Reprod. 1999;14(8):2149-54.
77. Poirot C, Vacher-Lavenu MC, Helardot P, Guibert J, Brugières L, Jouannet P. Human ovarian tissue cryopreservation: indications and feasibility. Hum Reprod. 2002;17(6):1447-52.
78. Schmidt KL, Byskov AG, Nyboe Andersen A, Müller J, Yding Andersen C. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. Human reproduction (Oxford, England). 2003;18(6):1158-64.
79. Gook DA, Edgar DH, Stern C. Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1, 2-propanediol. Human reproduction (Oxford, England). 1999;14(8):2061-8.
80. Isachenko V, Isachenko E, Woriedh M, Lapidus I, Weiss JM. Vitrification and conventional freezing of human ovarian tissue: Follicles formation, hormone production and gene expression. Molecular human reproduction. 2009;24:i16.
81. Tao T, Del Valle A. Human oocyte and ovarian tissue cryopreservation and its application. Journal of assisted reproduction and genetics. 2008;25(7):287-96.
82. Rodrigues RB, Uczay M, Brito VB, Godoy AC, Moura DJ, Vogel C, et al. Oxidative Stress and DNA Damage of Zebrafish Sperm at Different Stages of the Cryopreservation Process. Zebrafish. 2021;18(2):97-109.
83. Pereira BC, Ortiz I, Dorado J, Consuegra C, Diaz-Jimenez M, Demyda-Peyras S, et al. Evaluation of DNA Damage of Mare Granulosa Cells Before and After Cryopreservation Using a Chromatin Dispersion Test. J Equine Vet Sci. 2019;72:28-30.
84. Chetverikova EP. [DNA damage by active oxygen species in cryopreservation and the antioxydative properties of cryoprotectants]. Biofizika. 2012;57(2):368-76.
85. Adashi EY. Endocrinology of the ovary. Human reproduction (Oxford, England). 1994;9(5):815-27.
86. Ouni E, Bouzin C, Dolmans MM, Marbaix E, Pyr Dit Ruys S, Vertommen D, et al. Spatiotemporal changes in mechanical matrisome components of the human ovary from prepuberty to menopause. Human reproduction (Oxford, England). 2020;35(6):1391-410.

87. Wang S, Zheng Y, Li J, Yu Y, Zhang W, Song M, et al. Single-Cell Transcriptomic Atlas of Primate Ovarian Aging. Cell. 2020;180(3):585-600.e19.
88. Meryman HT. Cryoprotective agents. Cryobiology. 1971;8(2):173-83.
89. Elliott GD, Wang S, Fuller BJ. Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology. 2017;76:74-91.
90. Hagedorn J, Alkurdi G, Barker SA, Brunotte R, Deeb T, Hubenia O, et al. The technology in cryotechnology. Cryo letters. 2023;44(6):314-26.
91. Murray KA, Gibson MI. Chemical approaches to cryopreservation. Nat Rev Chem. 2022;6(8):579-93.
92. Awan M, Buriak I, Fleck R, Fuller B, Goltsev A, Kerby J, et al. Dimethyl sulfoxide: a central player since the dawn of cryobiology, is efficacy balanced by toxicity? Regen Med. 2020;15(3):1463-91.
93. Hovatta O. Methods for cryopreservation of human ovarian tissue. Reproductive biomedicine online. 2005;10(6):729-34.
94. Tuten Sevim E, Arat S. Combining dimethyl sulphoxide (DMSO) with different cryoprotectants ensures better cartilage cell cryopreservation. Cryo Letters. 2021;42(4):220-6.
95. Liu M, Chen C, Liang L, Yu C, Guo B, Zhang H, et al. A biocompatible cell cryoprotectant based on sulfoxide-containing amino acids: mechanism and application. J Mater Chem B. 2023;11(11):2504-17.
96. Whaley D, Damyar K, Witek RP, Mendoza A, Alexander M, Lakey JR. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. Cell Transplant. 2021;30:963689721999617.
97. von Bomhard A, Elsässer A, Ritschl LM, Schwarz S, Rotter N. Cryopreservation of Endothelial Cells in Various Cryoprotective Agents and Media - Vitrification versus Slow Freezing Methods. PLoS One. 2016;11(2):e0149660.
98. Oldenhof H, Bigalk J, Hettel C, de Oliveira Barros L, Sydykov B, Bajcsy Á C, et al. Stallion Sperm Cryopreservation Using Various Permeating Agents: Interplay Between Concentration and Cooling Rate. Biopreserv Biobank. 2017;15(5):422-31.
99. El Cury-Silva T, Nunes MEG, Casalechi M, Comim FV, Rodrigues JK, Reis FM. Cryoprotectant agents for ovarian tissue vitrification: Systematic review. Cryobiology. 2021;103:7-14.
100. Hovatta O, Silye R, Krausz T, Abir R, Margara R, Trew G, et al. Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. Hum Reprod. 1996;11(6):1268-72.
101. Kasai M, Niwa K, Iritani A. Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos. J Reprod Fertil. 1981;63(1):175-80.

102. Toishibekov YM, Kazybayeva AS, Assanova YA, Nurkenov TT, Toishybek DY. 44 The effect of various cryoprotective agents and slow cooling rate on viability of goat ovarian tissue. Reprod Fertil Dev. 2021;34(2):257.

3.8. APPENDIX

Figure

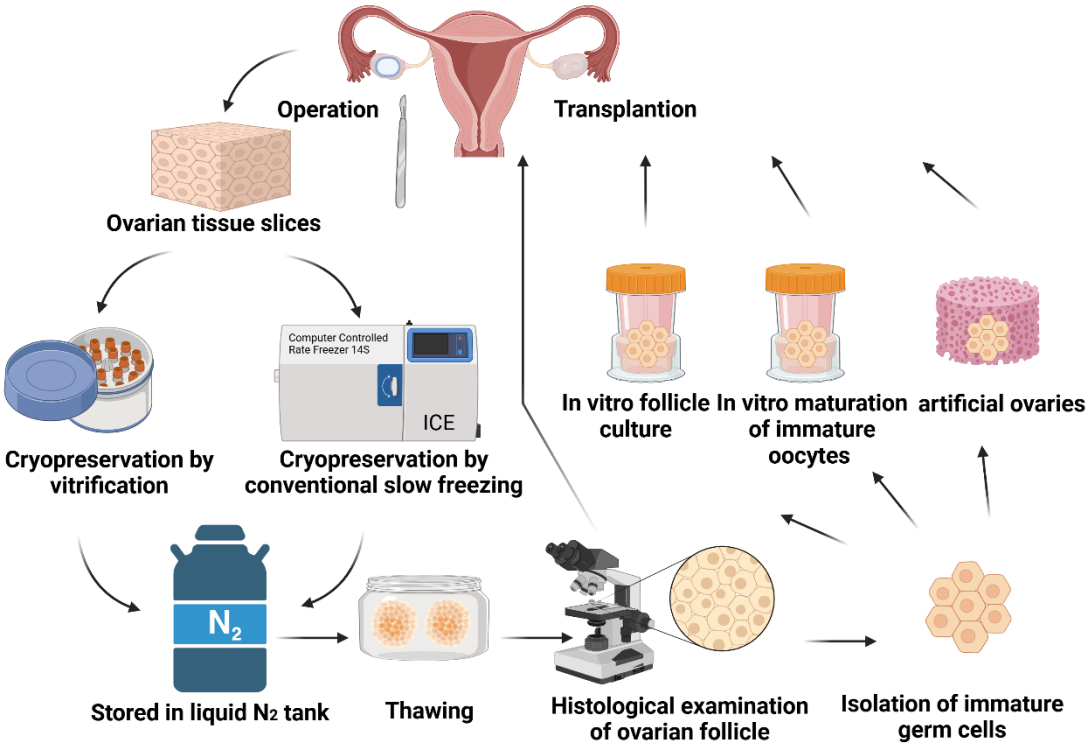

Figure 1. Schematic overview of ovarian cryopreservation

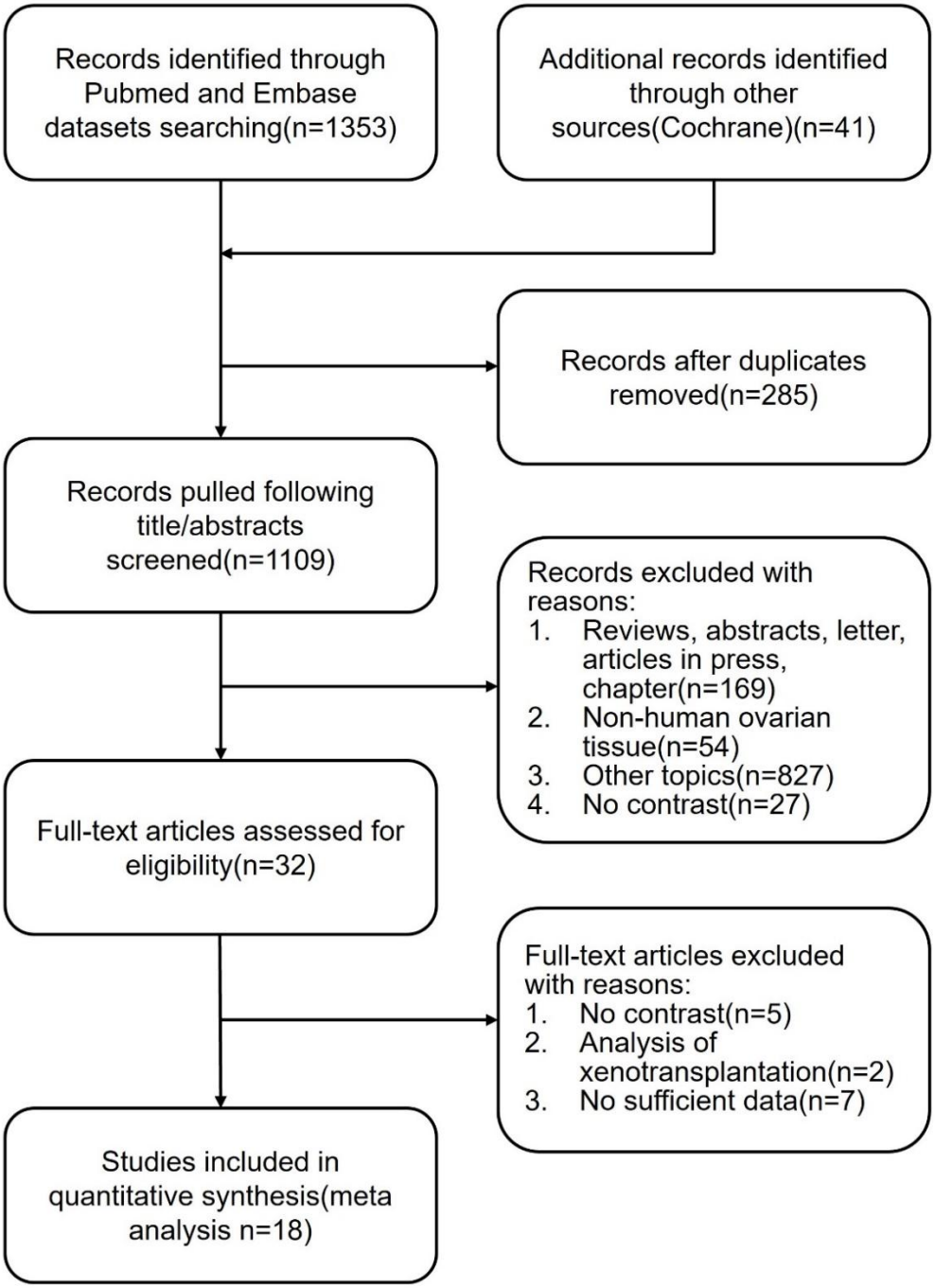

Figure 2. Flow diagram describing the research selection progress

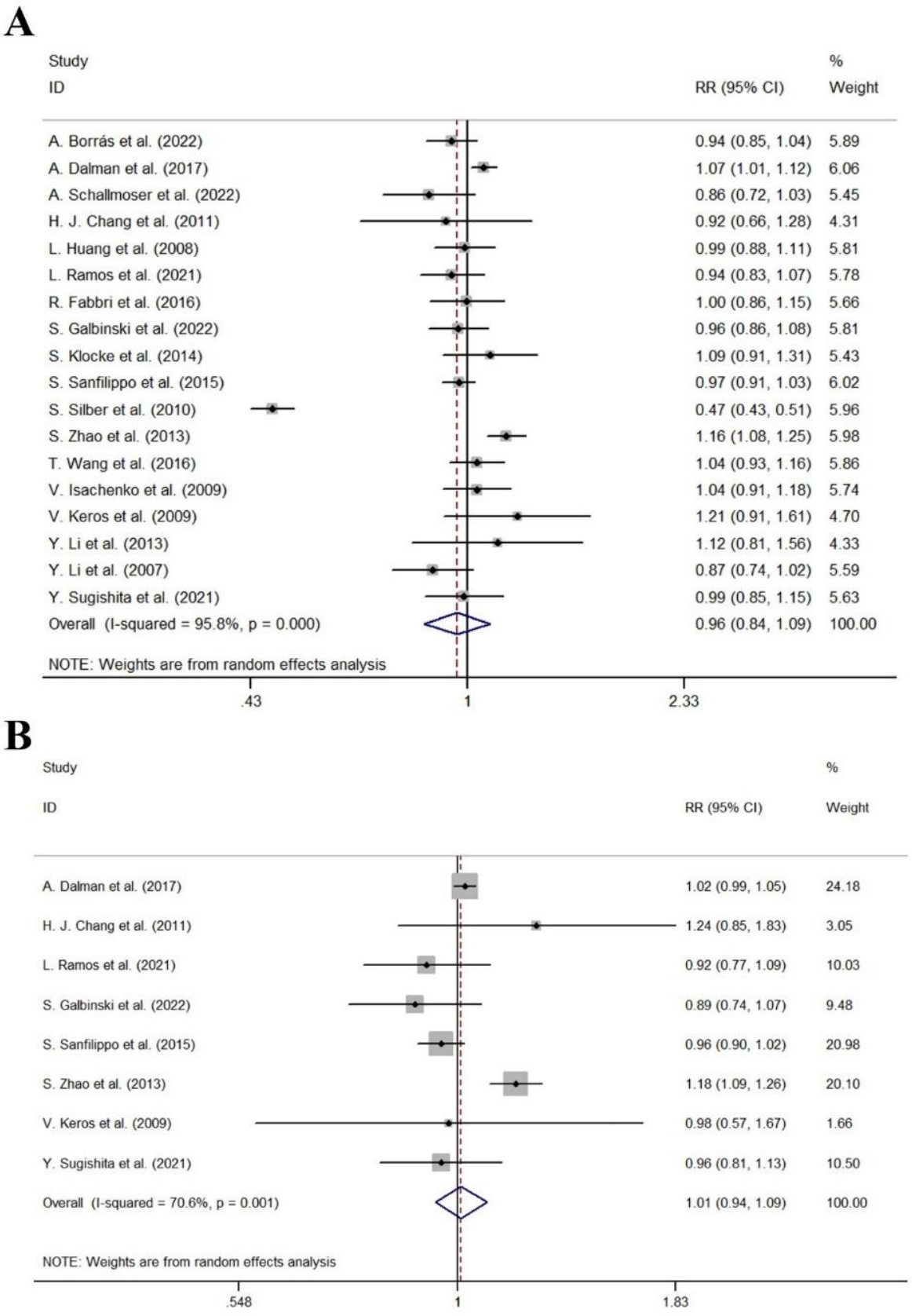

Figure 3. Forest plot for primary outcome A. Forest plot for the follicular viability of traditional slow cryopreservation and vitrification B. Forest plot for the proportion of intact primordial follicles of traditional slow cryopreservation and vitrification

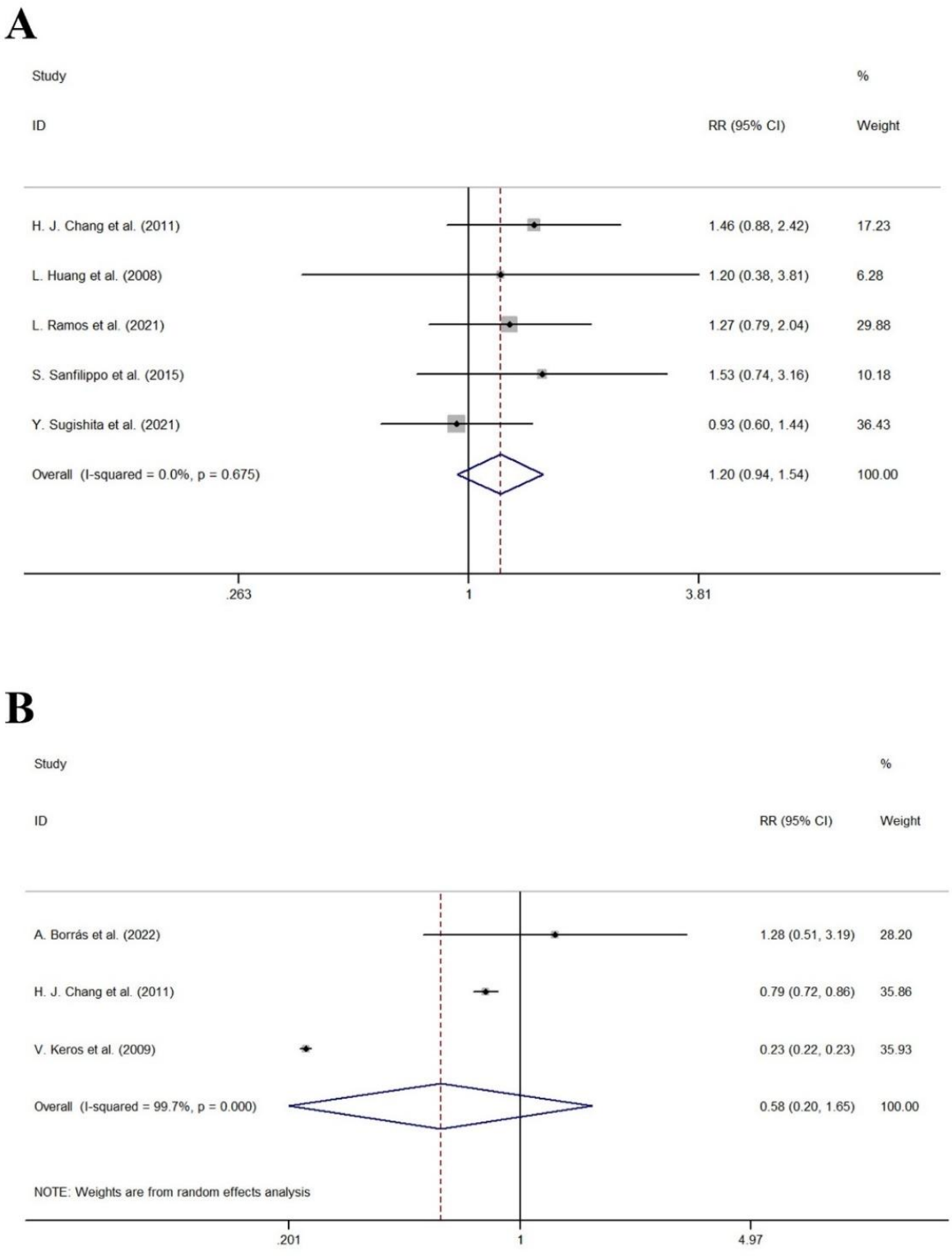

Figure 4. Forest plot for second outcome A. Forest plot for the proportion of DNA fragmented follicles of traditional slow cryopreservation and vitrification B. Forest plot for the proportion of stromal cells of traditional slow cryopreservation and vitrification

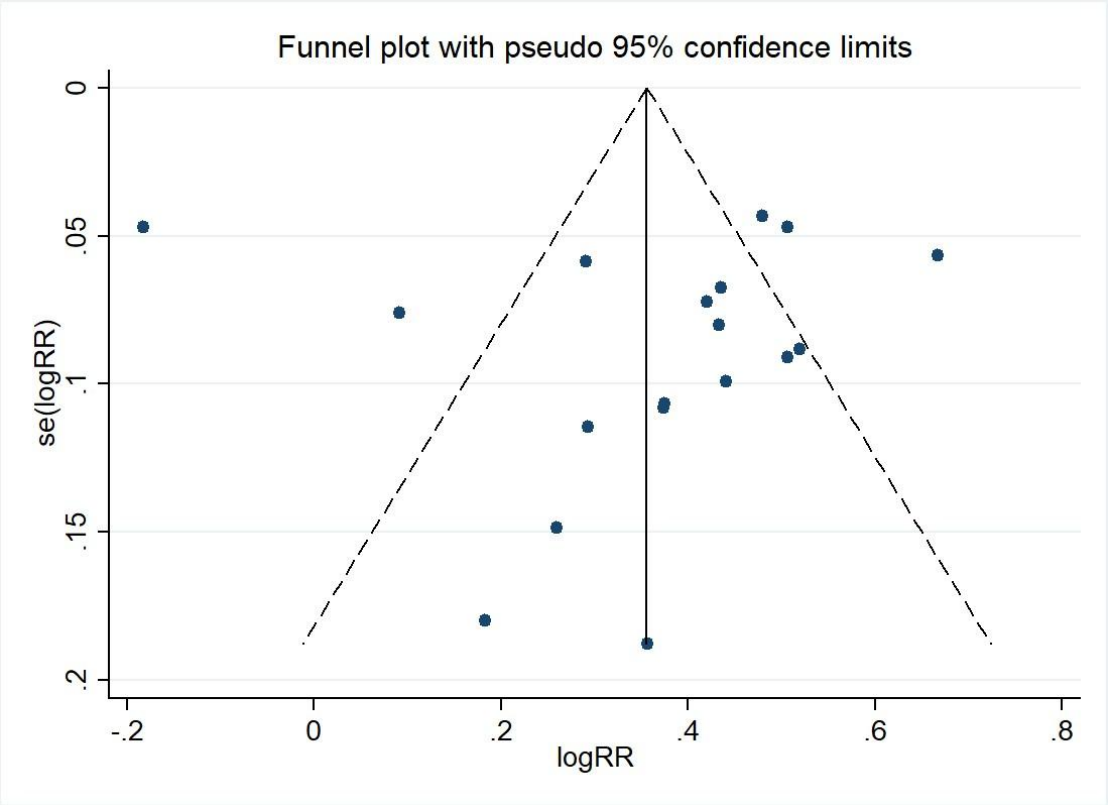

Figure 5. Funnel plot bias of the studies included in this meta-analysis

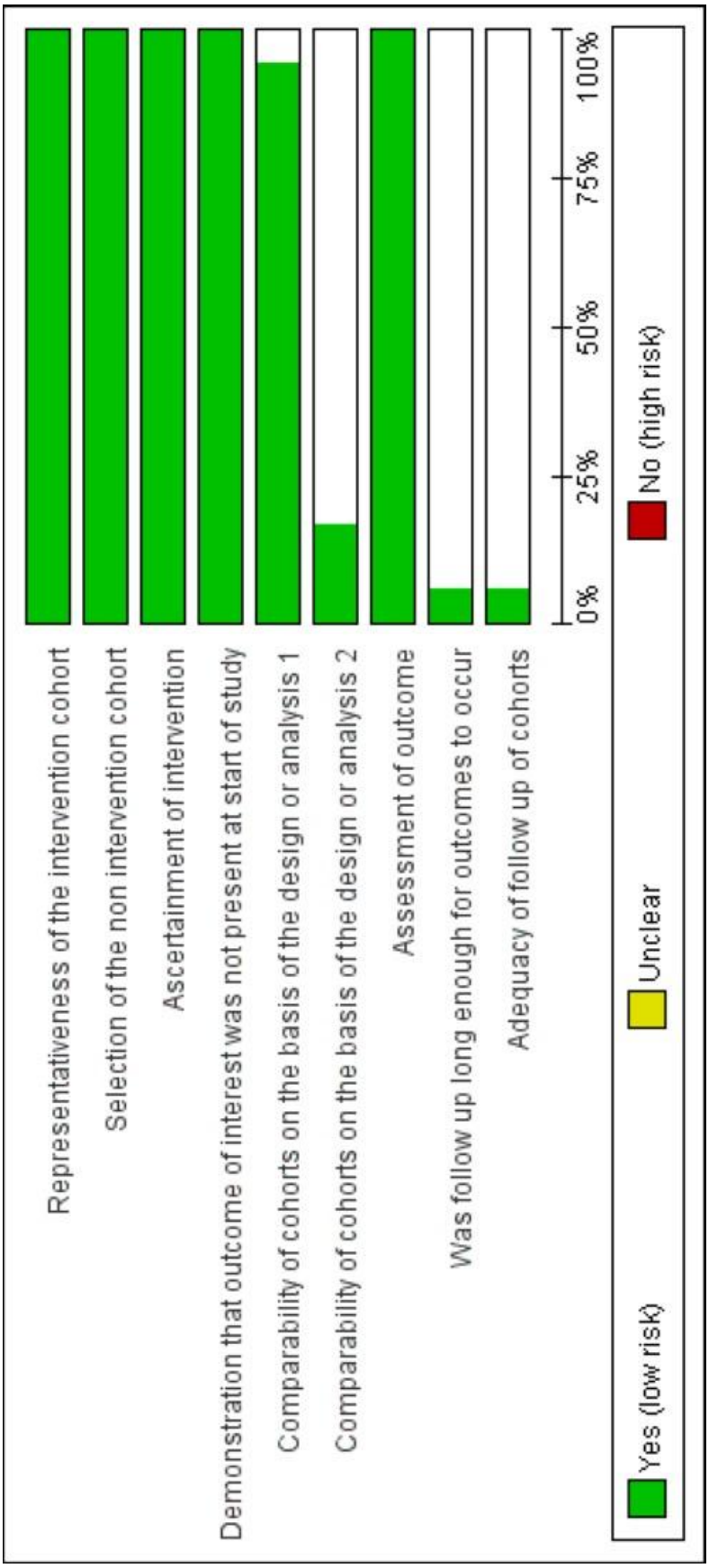

Figure 6. Risk of bias graph presenting authors' judgments according to Newcastle-Ottawa Scale for evidence-based medicine checklist about each risk of bias item as percentages across all included studies

Table 1 Main characteristics of the selected researches

Author	Publication year	Study region	Patients	Age range	Mean Age±SD	Surgical techniques	Type of surgery	follicles quality evaluation
A. Borrás et al.	2022	Spain	18	20-40	26.6 ± 5.5	Gender affirming surgery	transgender surgery	Light microscopy Live/dead cell viability assays Transmission electron microscopy
S. Galbinski et al.	2022	Brazil	12	NA	34.6±3.2	Laparoscopy	NA	Light microscope
Y. Sugishita et al.	2021	USA	5	NA	31±6.62	NA	NA	Microscope Immunohistochemistry
L. Huang et al.	2008	China	26	25-37	29.9±3.4	Laparoscopy or laparotomy	NA	Microscope TUNEL Assay
L. Ramos et al.	2021	Brazil	9	29-39	34.6±3	Laparoscopy	different gynecological conditions	Microscope
S. Silber et al.	2010	USA	16	24-40	32.8±5.7	Laparoscopy or minilaparotomy	unilateral oophorectomy after POF	Vability testing(type I collagenase) Microscope Transmission electron microscope
A. Schallmoser et al.	2022	Germany	30	14-41	26.7	Laparoscopy	prior initiation of gonadotoxic cancer treatment or due to a disease that causes premature limitation of the ovarian reserve	Vability testing(type I collagenase) Microscope
S. Zhao et al.	2013	China	15	23-45	33±5.1	NA	Cervical cancer; Endometrial cancer; Benign ovarian tumors	Light microscope Immunohistochemistry
V. Isachenko et al.	2009	Germany	15	18-33	23.1±4.9	NA	NA	Light microscope
T. Wang et al.	2016	China	11	21-49	31.8±8.36	Laparoscopic surgery	gynecological disease	Light microscope
Y. Li et al.	2013	China	20	19-45	33.2±9.5	Laparoscopy or laparotomy	different gynecological disease	Live/dead fluorescence analysis
Y. Li et al.	2007	China	15	22-37	33.1±2.9	Laparoscopy or laparotomy	benign ovarian cysts	Light microscope
R. Fabbri et al.	2016	Italy	6	14-34	24.5±9.3	Laparoscopy	Hodgkin lymphoma; breast cancer; brain tumour; medulloblastoma	Light and transmission electron microscopy
H. J. Chang et al.	2011	Korea	11	20-41	31.9±7.0	Laparoscopy	benign ovarian cysts or cesarean section	Microscope TUNEL Assay
A. Dalman et al.	2017	Iran	7	27-38	NA	NA	elective caesarean section	Light microscope
S. Klocke et al.	2014	Germany	21	NA	29.9 ± 5.0	Cesarean Section or abdominal surgery	elective cesarean section	Light microscope
S. Sanfilippo et al.	2015	France	5	NA	28.0 ± 1.1	Laparoscopy	benign cysts	Light microscope Cell Death Detection Kit
V. Keros et al.	2009	Sweden	20	28-43	33.3±4.0	NA	caesarean sections	Light microscope

Table 2 Main method and outcome of the selected researches

Author	freezing solution		follicular viability(percentage)		intact primordial follicle(percentage)		DNA fragmentation evaluation(percentage)		Follicular density	
	TSC	VC	TSC	VC	TSC	VC	TSC	VC	TSC	VC
A. Borrás et al.	1.5M PrOH + 0.2M sucrose solution in 1.8 ml cryovials	20% ethylene glycol, 20% DMSO and 0.5M sucrose in 1.8 ml cryovials	66.9	71.3	NA	NA	NA	NA	1.29±1.08 folls/mm2	1.2± 0.8 folls/mm2
S. Galbinski et al.	0.1 mol/L sucrose + 1.5 mol/L ethylene glycol	15% ethylene glycol and DMSO	75	54.5	70.3	79	NA	NA	NA	NA
Y. Sugishita et al.	1.5M DMSO + 0.1M sucrose + 10% HSA	10% ethylene glycol+20% serum substitute supplement	77.6	79.6	77.4	80.6	28.7	30.9	NA	NA
L. Huang et al.	1.5 M DMSO and 0.1 M sucrose solution in 1.8 ml cryovials	20% DMSO + 20% EG	84.3	85.3	NA	NA	6.1	5.3	NA	NA
L. Ramos et al.	0.1 M sucrose +1.5 M ethylene glycol	7.5% EG and 7.5%Dimethyl Sulfoxide	77.1	81.9	75	81	22.9	18.1	NA	NA
S. Silber et al.	1.5 mol/L 1,2-propanediol and 0.1 mol/L sucrose	7.5% EG and 7.5%Dimethyl Sulfoxide	41.7	89.1	NA	NA	NA	NA	NA	NA
A. Schallmoser et al.	10% DMSO + 11% HSA	10% ethylene glycol+10% serum substitute supplement	67	77.9	NA	NA	NA	NA	NA	NA
S. Zhao et al.	10% DMSO + 10% EG + 6% PEG + 0. 5% BSA + 0. 5mol /L sucrose	15% DMSO + 15% EG + 6% PEG + 0. 2% BSA + 0. 5mol /L sucrose	82.9	71.3	86.8	73.8	NA	NA	NA	NA
V. Isachenko et al.	1.5 M DMSO + 0.1 M sucrose + 10% SSS	2.62 M DMSO, 2.60 M acetamide, 1.31 M propylene glycol, and 0.0075 M polyethylene glycol	83	80	NA	NA	NA	NA	12.0±6.2/mm3	11.9±4.6/mm3
T. Wang et al.	0.1 M sucrose +1.5 M ethylene glycol + 10% HSA	7.5% ethylene glycol+7.5% DMSO+20% HSA	77.3	74.5	NA	NA	NA	NA	NA	NA
Y. Li et al.	1.5M DMSO + 0.1M sucrose + 10% HSA	7.5% ethylene glycol+7.5% DMSO+20% HSA	80.1	76.4	NA	NA	NA	NA	NA	NA
Y. Li et al.	1.5M DMSO + 0.1M sucrose	2M DMSO + 0.1M sucrose	72.6	80.3	NA	NA	NA	NA	NA	NA
R. Fabbri et al.	1.26 mol/L 1,2-propanediol and 0.175 mol/L sucrose and 30%HSA	2 M propylene glycol+3 M ethylene glycol+0.2 M sucrose+15% HSA	54.7	54.9	NA	NA	NA	NA	4.12±5.65/mm2	3.84±5.36/mm2
H. J. Chang et al.	1.5M DMSO + 0.1M sucrose + 10% HSA	20% EG + 20% HSA	60	65.3	80	64	46.7	31.5	NA	NA
A. Dalman et al.	10% DMSO + 2% HSA	7.5% ethylene glycol+7.5% DMSO+20% HSA	97.4	91.4	99.5	97.5	NA	NA	NA	NA
S. Klocke et al.	1.5M DMSO + 0.1M sucrose + 10% HSA	7.5% ethylene glycol+7.5% DMSO+20% HSA	72.7	66.7	NA	NA	NA	NA	NA	NA
S. Santfilippo et al.	1.5 M PrOH and 0.025 M raffinose	1.5 M PrOH+1.5 M EG	80.7	83.6	80.2	83.9	31.3	20.8	0.5 follicles/mm2	0.6 follicles/mm2
V. Keros et al.	1.5 M PrOH and 0.1 M sucrose	1.4 M DMSO, 1.5 M PrOH, 1.5 M EG	64.8	53.4	51	53	NA	NA	NA	NA

4. Positive Effect of Elevated Thawing Rate for Cryopreservation of Human Ovarian Tissue: Transcriptomic Analysis of Fresh and Cryopreserved Cells

Qingduo Kong [1], Plamen Todorov [2], Cheng Pei [1], Evgenia Isachenko [1], Gohar Rahimi [1,3], Nina Mallmann-Gottschalk [4] and Volodimir Isachenko [1]*

1 Department of Obstetrics and Gynecology, Medical Faculty, Cologne University, 50931 Cologne, Germany; qingduokong@gmail.com (Q.K.); chengpei314@gmail.com (C.P.); evgenia.isachenko@uk-koeln.de (E.I.); gohar.rahimi@amedes-group.com (G.R.)
2 Institute of Biology and Immunology of Reproduction of Bulgarian Academy of Sciences (BAS),1113 Sofia, Bulgaria; plamen.ivf@gmail.com
3 Medizinisches Versorgungszentrum AMEDES für IVF- und Pränatalmedizin in Köln GmbH, 50968 Cologne, Germany
4 Department of Obstetrics and Gynecology, Medical Faculty, Essen University, 45147 Essen, Germany; nina.mallmann-gottschalk@uk-essen.de
* Correspondence: vladimir.isachenko@uk-koeln.de

4.1. Abstract

Ovarian tissue cryopreservation has been gradually applied. It is essential to elucidate the differences between cryopreserved and fresh ovarian tissue and to refine cryopreservation protocols for improved outcomes. To explore the transcriptomic differences between fresh ovarian tissue and tissue cryopreserved with an elevated thawing rate. Ovarian tissue samples were collected and cryopreserved (frozen and thawed) following RNA sequencing and histological evaluation. Three groups were formed: fresh tissue (Group 1), frozen tissue after quick thawing at 100 °C (Group 2), and frozen tissue after slow thawing at 37 °C (Group 3). KEGG analysis showed that in comparison with Group 1, DEGs in Group 2 were mainly enriched in the cortisol synthesis and ovarian steroidogenesis pathways, and DEGs in the cells of Group 3 were mainly enriched in the ovarian steroidogenesis pathway. GO analysis showed that compared to cells of Group 2, DEGs in Group 3 were primarily enriched in the SRP-dependent co-translational protein targeting pathway and co-translational protein targeting to the membrane. The results were formulated with a minimal difference in the histological evaluation of cells after quick and slow thawed tissue. Cryopreservation of ovarian tissue by the described method does not decrease follicle production but downregulates the ovarian steroidogenesis pathway, reducing estrogen and progesterone secretion. The quick thawing of ovarian tissue increases the proliferation and apoptosis pathways of cells.

Keywords:

human ovarian tissue; cryopreservation; thawing; RNA sequencing; transcriptomics; differentially expressed genes (DEGs); Kyoto encyclopedia of genes and genomes (KEGG); gene ontology (GO)

4.2. Introduction

With advancements in science and technology, an increasing number of females are becoming aware of fertility preservation options. Fertility preservation is particularly relevant for females undergoing cancer treatment or those diagnosed with premature ovarian insufficiency (POI) [1,2,3]. In addition to oocyte cryopreservation and embryo cryopreservation, ovarian tissue cryopreservation (OTC) offers a more comprehensive approach to preserving ovarian function. This technique has gained significant attention and has become an important strategy for safeguarding female fertility [4,5,6,7]. However, there remains concern regarding whether cryopreserved ovarian tissue can maintain functionality comparable to that of fresh ovarian tissue. OTC has been gradually applied, and so far, more than 200 live births have been achieved, demonstrating its ability to restore fertility by producing functional oocytes [8,9]. Nevertheless, it is essential to elucidate the molecular and functional differences between cryopreserved and fresh ovarian tissue and to refine cryopreservation protocols for improved outcomes.

Currently, the primary techniques for ovarian tissue cryopreservation include conventional slow freezing and vitrification [10,11,12]. Conventional slow freezing is the most widely utilized method for ovarian tissue preservation. This technique systematically decreases the temperature of ovarian tissue to cryogenic levels using a computer-controlled programmable freezer. The critical factor in this method is the precise control of the cooling rate, typically set at 0.3 °C per minute, which minimizes the formation of intracellular and extracellular ice crystals, thereby preserving the integrity of cellular membranes and the overall tissue architecture [13,14]. Vitrification is an ultrarapid freezing technique that utilizes extremely high cooling rates to convert intracellular and extracellular fluids into a glass-like, amorphous solid state, effectively preventing ice crystal formation [15,16]. However, vitrification is less effective for larger tissue volumes, as the temperature gradient between the tissue surface and core can result in asynchronous cooling and potential tissue damage. Consequently, this study selected the more established method of slow freezing.

In addition to the freezing process, the thawing phase is equally critical in ovarian tissue cryopreservation. It is essential to restore the tissue to its pre-freezing state to the greatest extent possible. Primarily, preventing thermal damage to the tissue during thawing is the most important. Conventionally, the tissue is first placed at room temperature for 30 s, followed by immersion in 37 °C warm water until all ice has completely melted. This method minimizes thermal injury to the tissue during thawing. Notably, the first successful live birth resulting from ovarian cryopreservation in Germany employed this thawing method [14,17]. However, it is

also imperative to minimize the contact time between the tissue and the cryoprotectant during thawing. Reducing the exposure time to the cryoprotectant while ensuring that the tissue does not experience thermal damage can mitigate the cytotoxic effects of the cryoprotectant on ovarian tissue. During the thawing stage, the cell membrane is particularly vulnerable, and the osmotic pressure across the membrane is unbalanced. Therefore, achieving a rapid transition to the recovery phase is critical for maintaining cellular viability. The study group of the Medical Faculty of Cologne University investigated a method involving the exposure of cryovials to room temperature for 30 s, followed by immersion in a boiling water bath (100 °C) for 60 to 75 s. Moreover, there was precise monitoring of the ice melting, with the immersion time visually controlled: once the ice at the tip of the cryovial was reduced to approximately 1 mm in thickness, the cryovial was removed from the water. This technique minimizes the overall thawing time and the contact time of the tissue with the cryoprotectant while ensuring that thermal damage is avoided.

Consequently, this study used transcriptomic analysis to evaluate the differences between conventional slow freezing and fresh human ovarian tissue, as well as the effects of different thawing rates on ovarian tissue to better optimize ovarian cryopreservation techniques.

4.3. Results

4.3.1. Differential Expression Genes (DEGs)

Volcano plots were initially generated to compare upregulated and downregulated differentially expressed genes across tissues from different groups. In comparison to Group 1 (fresh ovarian tissue), the expression of 1839 genes was upregulated and 3740 genes were downregulated in Group 2 plus Group 3 (Cryopreserved ovarian tissue) as shown in Figure 1A. The expression of 314 genes was upregulated, and 2301 genes were downregulated in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) compared with Group 1 (shown in Figure 1B). At the same time, in Group 3 (frozen ovarian tissue after slow thawing at 37 °C), compared to Group 1, the expression of 722 genes was upregulated, while 3036 genes were downregulated as shown in Figure 1C. Compared to Group 2, Group 3 exhibited upregulation in 1502 genes and downregulation in 1109 genes (shown in Figure 1D).

4.3.2. Kyoto Encyclopedia of Genes and Genomes (KEGG)

KEGG pathway enrichment analysis based on DEGs was conducted. In comparison to Group 1 (fresh ovarian tissue), DEGs in Group 2 plus Group 3 (cryopreserved ovarian tissue) were

mainly enriched in the lysosome pathway as shown in Figure 2A. Compared to Group 1, DEGs in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) were mainly enriched in the cortisol synthesis pathway and ovarian steroidogenesis pathway, while DEGs in Group 3 (frozen ovarian tissue after slow thawing at 37 °C) were mainly enriched in the ovarian steroidogenesis pathway (Figure 2B,C). Compared to Group 2, DEGs in Group 3 were primarily enriched in the PI3K-Akt signaling pathway and ECM-receptor interaction pathway (shown in Figure 2D).

4.3.3. Gene Set Enrichment Analysis (GSEA)

GSEA is a type of in-depth data analysis based on predefined gene sets in the KEGG database. In comparison to Group 1 (fresh ovarian tissue), the enrichment of genes involved in the lysosome pathway in downregulation in Group 2 plus Group 3 (cryopreserved ovarian tissue) (Figure 3A). It was shown that the expression of the lysosomal pathway in cryopreserved ovarian tissue was significantly reduced compared to that in fresh ovarian tissue. Compared to Group 1, Group 2 (frozen ovarian tissue after quick thawing at 100 °C) showed downregulation of genes involved in the cortisol synthesis pathway (Figure 3B,C) and ovarian steroidogenesis pathway, while Group 3 (frozen ovarian tissue after slow thawing at 37 °C) showed downregulation of genes involved in the ovarian steroidogenesis pathway (Figure 3D). It was shown that both quick-thawing and slow-thawing cryopreserved ovarian tissues exhibited a significant reduction in the expression of the ovarian steroidogenesis pathway compared to fresh ovarian tissue. Compared to Group 3, Group 2 showed downregulation of genes involved in the PI3K-Akt signaling pathway and ECM-receptor interaction pathway (Figure 3E,F). It was shown that quick-thawing cryopreserved ovarian tissues exhibited downregulation of genes in the expression of the PI3K-Akt signaling pathway and ECM-receptor interaction pathway compared to slow-thawing cryopreserved ovarian tissues.

4.3.4. Gene Ontology (GO)

GO analysis based on DEGs was conducted. The analysis covered the functions of the biological process (BP), cellular component (CC), and molecular function (MF). In comparison to Group 1 (fresh ovarian tissue), DEGs in Group 2 plus Group 3 (cryopreserved ovarian tissue) were mainly enriched in neutrophil-mediated immunity and granulocyte activation as shown in Figure 4A. Compared to Group 1, DEGs in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) were mainly enriched in neutrophil-mediated immunity and neutrophil activation involved in immune response, while DEGs in Group 3 (frozen ovarian tissue after slow thawing at 37 °C) were mainly enriched in cellular response to the metal ion and humoral immune

response ovarian steroidogenesis pathway (Figure 4B,C). Compared to Group 2, DEGs in Group 3 were primarily enriched in the SRP-dependent co-translational protein targeting pathway and co-translational protein targeting to the membrane (shown in Figure 4D).

4.3.5. Morphology of Ovarian Tissue

Hematoxylin-Eosin (HE)-staining of fresh ovarian tissue is shown in Figure 5A,B. To minimize ice crystal formation during cryopreservation, the ovarian medulla was removed prior to the procedure. The fresh ovarian tissue in this study served as a control for the cryopreserved ovarian tissue, with the ovarian medulla also removed. Consequently, the HE-staining result showed the absence of mature follicles and secondary oocytes, revealing only a limited number of primordial follicles. The primordial follicle was encased by a layer of flat granulosa cells, which were essential for supporting oocyte development and for synthesizing and secreting hormones in the future. Additionally, stromal cells surrounding the follicles provided crucial support and nutrition for their further development. Similar results were also shown in the quick-thawing cryopreserved ovarian tissue in Figure 5C,D and in the slow-thawing cryopreserved ovarian tissue in Figure 5E,F. Clear primordial follicles, along with the surrounding granulosa cells and stromal cells, demonstrated that there was a minimal difference in HE-staining between quick-thawing cryopreserved ovarian tissue, slow-thawing cryopreserved ovarian tissue, and fresh ovarian tissue.

4.4. Discussion

4.4.1. Transcriptomic Differences Between Cryopreserved Ovarian Tissue and Fresh Ovarian Tissue

The primary function of ovarian tissue is the production of oocytes, enabling female fertility. Additionally, it secretes estrogen and progesterone, which are essential for the development and maturation of follicles. In recent years, ovarian cryopreservation technology has advanced significantly, offering improved methods for preserving female fertility. However, no studies have yet investigated the transcriptomic differences between cryopreserved ovarian tissue and fresh ovarian tissue. This study aims to investigate the transcriptomic differences between cryopreserved and fresh ovarian tissue.

Additionally, different thawing rates for cryopreserved ovarian tissue may lead to subtle differences in the ovarian tissue. The traditional thawing method is to leave the cryotube with the fragments of ovarian tissue at room temperature for 30 s and subsequently place it in 37 °C

warm water for 1 min until the ice has completely melted. Many studies have demonstrated its effectiveness, and, at the same time, the Cologne study group recommended leaving the cryotube with the fragments of ovarian tissue at room temperature for 30 s and then immersing for a period of 60 to 75 s in a water bath with boiling water (100 °C). When the ice at the tip is thinner than ~1 mm the cryotube should be removed as a quick thawing method. This method aligns with the principle of cryobiology. Any organism cryopreserved using existing cryopreservation techniques should be thawed as quickly as possible to minimize the duration of exposure to cryoprotectants, thereby reducing excessive dehydration and osmotic imbalance, and minimizing cell damage. However, the effectiveness of this quick thawing method requires further investigation. This study aims to explore whether different thawing rates produce significant differences in transcriptomic profiles compared to fresh ovarian tissue.

4.4.1.1. Differentially Expressed Genes (DEGs)

Identifying DEGs is a fundamental step in transcriptomic analysis, aimed at understanding the extent to which gene expression differences respond to specific conditions changes. The results from our experiment indicate significant differences in gene expression between cryopreserved ovarian tissue and fresh ovarian tissue, with over 5000 differentially expressed genes. Furthermore, more than 2000 genes exhibited significant changes between quick thawing cryopreserved ovarian tissue and fresh ovarian tissue, while over 3000 genes were identified as differentially expressed between slow-thawing cryopreserved ovarian tissue and fresh ovarian tissue. It demonstrates that, regardless of the thawing rate used, significant transcriptomic changes exist between cryopreserved ovarian tissue and fresh ovarian tissue.

In the comparison between cryopreserved ovarian tissue and fresh ovarian tissue, the genes that were significantly upregulated in cryopreserved ovarian tissue included FOSB and JUN. FOXB is a member of the Forkhead Box (FOX) family of transcription factors, while JUN forms heterodimers with FOSB to create the AP-1 transcription factor. This factor plays a critical role in important signaling pathways that regulate gene expression and which are involved in various physiological processes, including cell proliferation, apoptosis, stress response, and inflammation [18,19]. It suggests that a complex interplay of proliferation and apoptosis processes is implicated in the cryopreservation process. The significantly downregulated gene in cryopreserved ovarian tissue included DHCR4, which is closely related to cholesterol synthesis [20,21]. Besides oocyte production, the primary function of ovarian tissue is the synthesis of estrogen and progesterone, both of which are vital steroid hormones. Consequently, cryopreservation may impact the secretion of these hormones. Given the extensive changes observed in gene expression, we conducted pathway enrichment analysis

to identify specific pathways that exhibit significant alterations between cryopreserved ovarian tissue and fresh ovarian tissue.

4.4.1.2. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) Analysis

We found that only one pathway exhibited significant changes in KEGG analysis between cryopreserved ovarian tissue and fresh ovarian tissue, specifically the lysosomal pathway. Combined with GSEA, it was further confirmed that the lysosomal pathway is downregulated in the cryopreserved group. The lysosomal pathway is a crucial degradation pathway within cells, primarily responsible for processing cellular waste. This pathway plays an essential role in maintaining cell homeostasis, energy metabolism, signal transduction, and immune responses. Downregulation of the lysosomal pathway impairs the cell's ability to respond to stress, resulting in diminished lysosomal function, a reduced number of lysosomes, decreased enzyme activity, accumulation of toxic substances within the cells, and potential induction of cell death [22,23,24]. Consequently, during the cryopreservation process, cells may activate stress responses, and the downregulation of the lysosomal pathway diminishes the tissues' ability to manage these stressors, leading to the accumulation of endotoxins and promoting cellular apoptosis. When faced with the downregulation of the lysosomal pathway, it is necessary to implement appropriate intervention measures during the cryopreservation process to enhance the characteristics of cryopreserved ovarian tissue to resemble those of fresh ovarian tissue; the cryopreserved ovarian tissue may be better than before. The studies indicate that the degradative function of lysosomes can be enhanced by activating the autophagy pathway [25]. Furthermore, the downregulation of the lysosomal function is frequently associated with elevated oxidative stress in cells [26]. Supplementation with antioxidants may alleviate oxidative stress-induced damage to lysosomes and indirectly enhance lysosomal function. More research is necessary to investigate the potential incorporation of these substances into the cryopreservation process to more effectively preserve the characteristics of cryopreserved ovarian tissue.

We found that in KEGG pathways analysis that changed significantly between quick-thawing cryopreserved ovarian tissue and fresh ovarian tissue were the cortisol synthesis and secretion pathway and the ovarian steroidogenesis pathway. At the same time, slow-thawing cryopreserved ovarian tissue and fresh ovarian tissue changed significantly, and this was also on the ovarian steroidogenesis pathway. Combined with GSEA, it can be confirmed further that the ovarian steroidogenesis pathway is downregulated in both the quick-thawing cryopreserved ovarian tissue group and the slow-thawing cryopreserved ovarian tissue group

compared with fresh ovarian tissue. The ovarian steroidogenesis pathway uses cholesterol as a raw material in theca cells and granulosa cells of the follicles and is catalyzed by a series of enzymes to gradually generate different steroid hormones, mainly estrogen and progesterone [27,28]. The primary goal of ovarian cryopreservation is to preserve ovarian function, which encompasses not only the production of oocytes but also the synthesis of estrogen and progesterone. However, transcriptomic analysis has revealed that the ovarian steroidogenesis pathway is significantly downregulated in the cryopreserved group, leading to a reduction in the synthesis of estrogen and progesterone. Considering the downregulation of this pathway, it should be better to give appropriate intervention measures during the cryopreservation process, such as adding follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the cryopreservation process to promote the synthesis of estrogen and progesterone. Following cryopreservation and thawing, in vitro culture can be conducted, during which FSH and LH can be supplemented to promote the synthesis of estrogen and progesterone. Currently, FSH and LH are also incorporated into in vitro culture to further enhance the growth and development of ovarian cells. This study demonstrates that the ovarian steroidogenesis pathway is compromised during the cryopreservation process. Adding FSH and LH serves not only to promote the growth and development of ovarian cells but also to compensate for the damage incurred due to the downregulation of the ovarian steroidogenesis pathway during the cryopreservation process.

4.4.1.3. Gene Ontology (GO) Analysis

It can be found that the pathways with significant changes in GO analysis between cryopreserved ovarian tissue and fresh ovarian tissue included inflammation-related pathways, such as the neutrophil-mediated immune response pathway and the granulocyte activation pathway. During the cryopreservation process, cells are exposed to environmental changes that trigger stress responses. Notably, inflammation-related responses differ between quick-thawing cryopreserved ovarian tissue and fresh ovarian tissue, as well as between slow-thawing cryopreserved ovarian tissue and fresh ovarian tissue. These changes are linked to the stress state of the cells and exhibit minimal correlation with ovarian cell development, fertility, proliferation, or apoptosis.

4.4.2. Transcriptomic Differences Between Quick-Thawing Cryopreserved Ovarian Tissue and Slow-Thawing Cryopreserved Ovarian Tissue

The differences between cryopreserved ovarian tissues with two thawing rates and fresh ovarian tissues are obvious. However, the distinctions between the two thawing rates

themselves remain unclear. For instance, the results of hematoxylin and eosin (HE) staining indicated that both thawing rates can generate viable follicles. However, whether quick thawing results in less damage to ovarian tissues compared to slow thawing requires further investigation. Studies have shown that quick thawing may offer several advantages. Firstly, it can reduce ice crystal formation by effectively preventing and minimizing the regrowth of ice crystals during the thawing process. In contrast, during slow thawing, residual cryoprotectants or water molecules may recrystallize, leading to the formation of ice crystals. These ice crystals can inflict physical damage to both intracellular and extracellular structures, including disrupting cell membranes and damaging organelles. Secondly, quick thawing helps preserve cell structure and function by maintaining the integrity of the cell membrane in ovarian tissue and protecting the functions of key cell types, such as follicles and granulosa cells. Prolonged exposure of cells to cryoprotectants during slow thawing can increase their toxicity, leading to cell membrane damage, metabolic disorders, and even cell death. Additionally, slow thawing may result in incomplete or unstable phase transitions of the cell membrane, particularly in the presence of cryoprotectants, which can further compromise membrane integrity and make it more fragile. Finally, quick thawing facilitates better osmotic pressure balance between the intracellular and extracellular environments. Prolonged resuscitation times can exacerbate damage to the membrane structure, negatively impacting cell integrity and function. Fluctuations in temperature during the resuscitation process, coupled with the removal of cryoprotectants, can disrupt osmotic pressure equilibrium. If the recovery period is excessively lengthy, osmotic pressure may not normalize swiftly, leading to cell dehydration or excessive hydration. This imbalance can cause cells to either swell or shrink, ultimately affecting their survival and functionality [29,30,31,32]. This study aims to investigate the transcriptomic differences between quick and slow thawing rates.

4.4.2.1. Differentially Expressed Genes (DEGs)

The results indicated that there are numerous differentially expressed genes in quick- and slow-thawing cryopreserved ovarian tissue, with over 2000 genes showing significant changes. This finding confirms that there are distinct transcriptomic differences between quick- and slow-thawing cryopreserved ovarian tissue. In the comparison between the quick-thawing group and the slow-thawing group, the gene that was significantly upregulated in the quick-thawing group was GADD45B, which is a member of the GADD45 family. Members of the GADD45 family are involved in various biological processes, including the cellular stress response, the regulation of proliferation, and DNA repair. Notably, GADD45B is particularly sensitive to a range of stressors and serves as a crucial regulator of cellular responses to environmental changes [33,34,35]. This finding indicates that the cellular stress response in the quick-thawing

group is more pronounced. When the cellular stress response is elevated, it can elicit a series of complex biological effects, both beneficial and detrimental. On the positive side, the stress response can activate cellular repair mechanisms, such as DNA repair, protein folding, and degradation, thereby aiding in the restoration of normal cellular function. It also promotes cell proliferation, differentiation, and immune activity. Conversely, an excessive stress response can result in the overproduction of reactive oxygen species (ROS), leading to oxidative damage that affects cell membranes, DNA, and proteins, as well as promoting apoptosis. Therefore, while an enhanced cellular stress response may confer protective effects, such as tissue repair and immune enhancement, excessive stress can cause cellular damage and apoptosis. Consequently, although the quick-thawing group elicited an increased stress response, further investigation is needed to determine whether this ultimately leads to enhanced cell proliferation or increased apoptosis. Because the number of genes changed was too high, we conducted pathway enrichment analysis to identify the specific pathways that exhibited significant changes between quick- and slow-thawing cryopreserved ovarian tissue.

4.4.2.2 Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) Analysis

It was identified in KEGG analysis between quick- and slow-thawing cryopreserved ovarian tissue that the pathways with significant changes included the PI3K-Akt signaling pathway and ECM-receptor interaction pathway. Combined with GSEA, it can be confidently confirmed that compared to slow-thawing cryopreserved ovarian tissue, the PI3K-Akt signaling pathway and ECM-receptor interaction pathway in the quick-thawing cryopreserved ovarian tissue were significantly downregulated. The PI3K-Akt signaling pathway plays a critical role in various biological processes, including cell growth, proliferation, survival, migration, and metabolism. It primarily promotes cell cycle progression, enhances cell proliferation, and increases cell survival by inhibiting apoptotic pathways [36,37]. The significant downregulation of this pathway may suggest that cell survival in the quick-thawing group is markedly lower than in the slow-thawing one. The ECM-receptor interaction pathway is crucial for cell signaling, proliferation, differentiation, and migration. Downregulation of this pathway may lead to decreased adhesion between cells and the extracellular matrix, negatively impacting cell morphology, migration, and proliferation [38,39,40]. This further substantiates that morphological changes in the quick-thawing group promote apoptosis. Consequently, it indicates that a series of apoptotic processes occurred in the quick-thawing group following the stress response.

4.4.2.3. Gene Ontology (GO) Analysis

It was identified in GO analysis between quick- and slow-thawing cryopreserved ovarian tissue that the pathways with significant changes included the SRP-dependent co-translational protein targeting pathway and co-translational protein targeting to the membrane. The SRP-dependent co-translational protein targeting pathway is a critical protein transport mechanism within the cell, primarily responsible for directing secretory proteins, membrane proteins, and proteins destined for organelles to the endoplasmic reticulum (ER) during synthesis. This process ensures that newly synthesized polypeptides are accurately directed as they are produced on ribosomes. Under stress conditions, upregulation of the SRP pathway may help cells synthesize and transport stress-related proteins more effectively. By enhancing the protein-processing capacity of the ER, upregulation of the SRP pathway can improve cell survival and reduce cell death. The co-translational targeting protein mechanism plays an important role in intracellular protein synthesis and transport. Through effective signal recognition and transport processes, cells can ensure the correct folding and function of proteins, thereby maintaining the normal operation and physiological function of cells. The upregulation of this pathway, together with the SRP pathway, promotes protein synthesis, improves cell survival, and reduces cell death [41,42,43]. Consequently, cells in the quick-thawing group exhibited heightened stress responses. Activation of the SRP-dependent pathway and the co-translational targeting pathway can enhance cell survival and mitigate cell death. In the KEGG pathway enrichment analysis, it was determined that the quick-thawing group facilitated the apoptotic process via the PI3K-Akt signaling pathway and ECM-receptor interaction pathway following the stress response. However, the GO results indicated that the quick-thawing group also promoted cell survival through the SRP-dependent pathway and the co-translational targeting pathway in response to stress. Thus, following the stress response, quick-thawing cryopreserved ovarian tissue exhibited both pro-apoptotic and pro-survival activities. Further investigation is required to determine which pathway has a stronger influence.

4.4.3. Limitations

This study aims to explore the transcriptomic differences between fresh ovarian tissue and tissue cryopreserved with an elevated thawing rate. Our results indicate that cryopreservation of ovarian tissue by the described method does not decrease follicle production but downregulates the ovarian steroidogenesis pathway, reducing estrogen and progesterone secretion. Quick thawing of ovarian tissue increases the proliferation and apoptosis pathways of cells. The main limitation of our study is the small number of ovarian tissue samples. As human ovarian tissue is difficult to obtain due to ethical and clinical constraints, this has

restricted the number of experimental samples for our study. Further studies are required to validate and expand upon the conclusions presented in this article.

4.5. Materials and Methods

4.5.1. Design of Experiments

A total of 9 human ovarian tissue samples was collected and divided into 3 groups. The technology in our studies was developed for tumor patients. The ovarian tissue fragments used for the experiments were obtained from patients involved in a fertility treatment program. They were all diagnosed with tumors (including Hodgkin's Lymphoma, Non-Hodgkin's Lymphoma, and Chronic Myeloid Leukemia). Informed consent was obtained from patients whose tissue was collected for this study. Ovarian tissue fragments were obtained from 3 patients aged 18, 26 and 27 (median age 23.7 years). Three tissue fragments from each patient were used for experiments. Three groups were formed: fresh tissue (Group 1), frozen tissue after quick thawing at 100 °C (Group 2), and frozen tissue after slow thawing at 37 °C (Group 3). Three samples were used in each group (Figure 6).

4.5.2. Collection and Cryopreservation of Ovarian Tissue

The study was conducted following the Declaration of Helsinki and approved by the Institutional Ethics Committee of Cologne University (applications 999,184 and 13-147) and by the Bulgarian Ethics Committee. Informed consent was obtained from patients whose ovarian tissue was collected for this study. All chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA) unless otherwise stated.

Cryopreservation of ovarian tissue was conducted following our previously published protocols [44,45,46,47]. On the day of freezing, ovarian tissue pieces were placed at room temperature in 20 mL of the freezing medium, consisting of basal medium supplemented with 6% dimethyl sulfoxide (DMSO), 6% ethylene glycol, and 0.15 M sucrose. The tissue pieces were then transferred into standard 5 mL cryo-vials (Thermo Fisher Scientific, Rochester, NY, USA), prefilled with the freezing medium, and frozen using the IceCube 14S freezer (SyLab, Neupurkersdorf, Austria). The cryopreservation protocol was as follows: (1) the starting temperature was −6 °C; (2) samples were cooled from −6 °C to −34 °C at a rate of 0.3 °C/min; and (3) at −34 °C, the cryo-vials were plunged into liquid nitrogen. The freezing protocol also included an auto-seeding step at −6 °C.

Quick thawing: Tissue thawing was achieved by placing the cryovials at room temperature for 30 s and then immersing them in a 100 °C (boiling water) water bath for 60 s and draining the vial contents into the solution to remove the cryoprotectant. The exposure time in boiling water was visually controlled by the presence of ice in the culture medium; the vials were removed from the boiling water once the ice reached a size of 2 to 1 mm, at which point the final temperature of the culture medium was between 4 and 10 °C. Within 5 to 10 s of thawing, the tissue fragments in the cryovials were drained into 10 mL of thawing solution (basal medium containing 0.5 M sucrose) in a 100 mL specimen container (Sarstedt, Neumbrecht, Germany). After exposure of the tissue to sucrose for 15 min, stepwise rehydration of the cells was performed as previously reported.

Slow thawing: This thawing method is the same as the quick-thawing method described above, except that the cryovials were immersed in a 37 °C water bath for 1 min to thaw the tissue until the ice completely melted.

4.5.3. Sequencing and Data Extraction

Each sample of ovarian tissue was used for RNA extraction with the Trizol method. RNA integrity was assessed using the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand of cDNA was synthesized using random hexamer primers. Then the second strand of cDNA was synthesized using dUTP, instead of dTTP. The directional library was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. After library quality control, different libraries were pooled based on the effective concentration and targeted data amount, then subjected to Illumina sequencing. The basic principle of sequencing is “Sequencing by Synthesis”, where fluorescently labeled dNTPs, DNA polymerase, and adapter primers are added to the sequencing flow cell for amplification. As each sequencing cluster extends its complementary strand, the addition of each fluorescently labeled dNTP releases a corresponding fluorescence signal. The sequencer captures these fluorescence signals and converts them into sequencing peaks through computer software, thereby obtaining the sequence information of the target fragment. Reference genome and gene model annotation files were downloaded from the genome website directly. The index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. We selected Hisat2 as the mapping tool for Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus obtain a better

mapping result than other non-splice mapping tools. The mapped reads of each sample were assembled by StringTie (v1.3.3b) in a reference-based approach. StringTie uses a novel network flow algorithm as well as an optional de novo assembly step to assemble and quantitate full-length transcripts representing multiple splice variants for each gene locus.

4.5.4. Differential Expression Analysis

For DESeq2 with biological replicates, differential expression analysis for two conditions/groups was performed using the DESeq2 R package (1.20.0). DESeq2 provides statistical programs for determining differential expression in digital gene expression data using models based on negative binomial distribution. The resulting p-value is adjusted using Benjamini and Hochberg's methods to control the error discovery rate. The corrected p-value $\leqslant$ 0.05 and |log2(foldchange)| $\geqslant$ 1 was set as the threshold of significant differential expression.

4.5.5. GO and KEGG Enrichment Analysis of Differentially Expressed Genes

GO enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected p-values less than 0.05 were considered significantly enriched by differentially expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used the cluster profile R package to test the statistical enrichment of differential expression genes in KEGG pathways.

4.5.6. Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is a computational approach to determine if a predefined Gene Set can show a significant consistent difference between two biological states. The genes were ranked according to the degree of differential expression in the two samples, and then the predefined Gene Set was tested to see if they were enriched at the top or bottom of the list. Gene Set Enrichment Analysis can include subtle expression changes. We use the local version of the GSEA analysis tool http://www.broadinstitute.org/gsea/index.jsp, GO, and KEGG data sets were used for GSEA independently.

4.5.7. Histological Analysis

Ovarian tissues were fixed in 3.5% paraformaldehyde for 24 h at 4 °C and then embedded in paraffin wax. Sections of 4 μm thickness were cut, with every 10th section mounted on glass slides and stained using hematoxylin and eosin. Morphological analysis of tissue development and viability was performed under a Nikon Diaphot 300 microscope at 200× and 400× magnification.

4.6. Conclusions

Cryopreservation of ovarian tissue by the described method does not decrease follicle production but downregulates the ovarian steroidogenesis pathway, reducing estrogen and progesterone secretion. Quick thawing of ovarian tissue increases the proliferation and apoptosis pathways of cells.

Author Contributions

Conceptualization, V.I. and Q.K.; methodology, E.I. and C.P.; software, Q.K. and C.P.; validation, Q.K., G.R. and N.M.-G.; formal analysis, C.P. and E.I.; investigation, G.R.; resources, V.I.; data curation, E.I. and Q.K.; writing—original draft preparation, Q.K. and C.P.; writing review and editing, V.I. and P.T.; visualization, E.I. and P.T.; supervision, V.I. and G.R.; project administration, N.M.-G. and V.I.; funding acquisition, Q.K. and C.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:

The study was conducted by the Declaration of Helsinki and approved by the Institutional Ethics Committee of Cologne University (applications 999,184 and 13-147) and by the Bulgarian Ethics Committee.

Informed Consent Statement:

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement:

The raw data of RNA-seq can be downloaded at “Sequence read archive” on National Center for Biotechnology Information (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1188025?reviewer=or17fgchi8hki9n00bv4e3n1b3, accessed on 12 December 2024).

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Conflicts of Interest:

Gohar Rahimi was employed by Medizinisches Versorgungszentrum AMEDES für IVF- und Pränatalmedizin in Köln GmbH. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.7. References

1. Oktay, K. Fertility preservation in patients with cancer: ASCO clinical practice guideline update. J. Clin. Oncol. 2018, 36, 1994–2001.[CrossRef] [PubMed]
2. Christianson, M.S.; Oktay, K. Advances in fertility-preservation surgery: Navigating new frontiers. Fertil. Steril. 2019, 112,438–445. [CrossRef] [PubMed]
3. Antunes, M.B.; Cardeal, S.P.; Magalhães, M.; Vale-Fernandes, E.; Barreiro, M.; Sá, R.; Sousa, M. Preservation of fertility in female patients with hematologic diseases. Blood Rev. 2023, 62, 101115. [CrossRef] [PubMed]
4. Vukovic´, P.; Kasum, M.; Oreškovic´, D.; C˘ ehic´, E.; Raguž, J.; Elezaj, S.; Beketic´-Oreškovic´, L. Importance of ovarian tissue cryopreservation in fertility preservation and anti-aging treatment. Gynecol. Endocrinol. Off. J. Int. Soc. Gynecol. Endocrinol. 2019,35, 919–923. [CrossRef]
5. Fraison, E.; Huberlant, S.; Labrune, E.; Cavalieri, M.; Montagut, M.; Brugnon, F.; Courbiere, B. Live birth rate after female fertility preservation for cancer or haematopoietic stem cell

transplantation: A systematic review and meta-analysis of the three main techniques; embryo, oocyte and ovarian tissue cryopreservation. Hum. Reprod. 2023, 38, 489–502. [CrossRef]
6. Dolmans, M.M.; Donnez, J.; Cacciottola, L. Fertility Preservation: The Challenge of Freezing and Transplanting Ovarian Tissue.Trends Mol. Med. 2021, 27, 777–791. [CrossRef]
7. Dolmans, M.M.; Donnez, J. Fertility preservation in women for medical and social reasons: Oocytes vs ovarian tissue. Best Pract.Res. Clin. Obstet. Gynaecol. 2021, 70, 63–80. [CrossRef]
8. Donnez, J.; Squifflet, J.; Jadoul, P.; Demylle, D.; Cheron, A.C.; Van Langendonckt, A.; Dolmans, M.M. Pregnancy and live birth after autotransplantation of frozen-thawed ovarian tissue in a patient with metastatic disease undergoing chemotherapy and hematopoietic stem cell transplantation. Fertil. Steril. 2011, 95, 1787.e1–1787.e4. [CrossRef]
9. Roux, C.; Amiot, C.; Agnani, G.; Aubard, Y.; Rohrlich, P.S.; Piver, P. Live birth after ovarian tissue autograft in a patient with sickle cell disease treated by allogeneic bone marrow transplantation. Fertil. Steril. 2010, 93, 2413.e15–2413.e19. [CrossRef]
10. Zhou, X.H. Comparison of vitrification and conventional slow freezing for cryopreservation of ovarian tissue with respect to the number of intact primordial follicles: A meta-analysis. Medicine 2016, 95, e4095. [CrossRef]
11. Kometas, M.; Christman, G.M.; Kramer, J.; Rhoton-Vlasak, A. Methods of Ovarian Tissue Cryopreservation: Is Vitrification Superior to Slow Freezing?-Ovarian Tissue Freezing Methods. Reprod. Sci. 2021, 28, 3291–3302. [CrossRef] [PubMed]
12. Herraiz, S. Improving ovarian tissue cryopreservation for oncologic patients: Slow freezing versus vitrification, effect of different procedures and devices. Fertil. Steril. 2014, 101, 775–784.e1. [CrossRef] [PubMed]
13. Vanni, V.S.; De Lorenzo, R.; Privitera, L.; Canti, V.; Viganò, P.; Rovere-Querini, P. Safety of fertility treatments in women with systemic autoimmune diseases (SADs). Expert Opin. Drug Saf. 2019, 18, 841–852. [CrossRef] [PubMed]
14. Isachenko, V.; Dittrich, R.; Keck, G.; Isachenko, E.; Rahimi, G.; van der Ven, H.; Montag, M.; Hoffmann, I.; Müller, A.; Distler, W.; et al. Cryopreservation of Ovarian Tissue: Detailed Description of Methods for Transport, Freezing and Thawing. Geburtshilfe Frauenheilkd 2012, 72, 927–932. [CrossRef]
15. Rahimi, G. Apoptosis in human ovarian tissue after conventional freezing or vitrification and xenotransplantation. CryoLetters 2009, 30, 300–309.
16. Amorim, C.A.; Curaba, M.; Van Langendonckt, A.; Dolmans, M.M.; Donnez, J. Vitrification as an alternative means of cryopreserving ovarian tissue. Reprod. Biomed. Online 2011, 23, 160–186. [CrossRef]
17. Müller, A.; Keller, K.;Wacker, J.; Dittrich, R.; Keck, G.; Montag, M.; Van der Ven, H.;Wachter, D.; Beckmann, M.W.; Distler,W. Retransplantation of cryopreserved ovarian tissue: The first live birth in Germany. Dtsch. Ärzteblatt Int. 2012, 109, 8–13. [CrossRef]

18. Kouzarides, T.; Ziff, E. Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding.Nature 1989, 340, 568–571. [CrossRef]
19. Gazon, H.; Barbeau, B.; Mesnard, J.M.; Peloponese, J.M., Jr. Hijacking of the AP-1 Signaling Pathway during Development of ATL. Front. Microbiol. 2017, 8, 2686. [CrossRef]
20. Filipovic, I.; Buddecke, E. Calmodulin antagonists suppress cholesterol synthesis by inhibiting sterol delta 24 reductase. Lipids 1987, 22, 261–265. [CrossRef]
21. Fernández, C.; Suárez, Y.; Ferruelo, A.J.; Gómez-Coronado, D.; Lasunción, M.A. Inhibition of cholesterol biosynthesis by Delta22- unsaturated phytosterols via competitive inhibition of sterol Delta24-reductase in mammalian cells. Biochem. J. 2002, 366, 109–119. [CrossRef] [PubMed]
22. Tan, J.X.; Finkel, T. A phosphoinositide signalling pathway mediates rapid lysosomal repair. Nature 2022, 609, 815–821. [CrossRef] [PubMed]
23. Wang, F.; Gómez-Sintes, R.; Boya, P. Lysosomal membrane permeabilization and cell death. Traffic 2018, 19, 918–931. [CrossRef] [PubMed]
24. Yang, C.;Wang, X. Lysosome biogenesis: Regulation and functions. J. Cell Biol. 2021, 220, e202102001. [CrossRef]
25. Levine, B.; Kroemer, G. Biological Functions of Autophagy Genes: A Disease Perspective. Cell 2019, 176, 11–42. [CrossRef]
26. Pellegrini, F.R.; De Martino, S.; Fianco, G.; Ventura, I.; Valente, D.; Fiore, M.; Trisciuoglio, D.; Degrassi, F. Blockage of autophagosome-lysosome fusion through SNAP29 O-GlcNAcylation promotes apoptosis via ROS production. Autophagy 2023, 19, 2078–2093. [CrossRef]
27. Li, L.; Shi, X.; Shi, Y.; Wang, Z. The Signaling Pathways Involved in Ovarian Follicle Development. Front. Physiol. 2021, 12, 730196.[CrossRef]
28. Wood, J.R.; Strauss, J.F., 3rd. Multiple signal transduction pathways regulate ovarian steroidogenesis. Rev. Endocr. Metab. Disord.2002, 3, 33–46. [CrossRef]
29. Hagedorn, J.; Alkurdi, G.; Barker, S.A.; Brunotte, R.; Deeb, T.; Hubenia, O.; Khayyat, D.; Leal-Marin, S.; Rittinghaus, T.; Glasmacher, B. The technology in cryotechnology. CryoLetters 2023, 44, 314–326. [CrossRef]
30. Whaley, D.; Damyar, K.; Witek, R.P.; Mendoza, A.; Alexander, M.; Lakey, J.R. Cryopreservation: An Overview of Principles and Cell-Specific Considerations. Cell Transplant. 2021, 30, 963689721999617. [CrossRef]
31. Pegg, D.E. Principles of cryopreservation. Methods Mol. Biol. 2007, 368, 39–57. [CrossRef] [PubMed]
32. Baust, J.M.; Campbell, L.H.; Harbell, J.W. Best practices for cryopreserving, thawing, recovering, and assessing cells. Vitr. Cell. Dev. Biol. Anim. 2017, 53, 855–871. [CrossRef] [PubMed]

33. Liebermann, D.A.; Hoffman, B. Gadd45 in the response of hematopoietic cells to genotoxic stress. Blood Cells Mol. Dis. 2007, 39, 329–335. [CrossRef] [PubMed]
34. Palomer, X.; Salvador, J.M.; Griñán-Ferré, C.; Barroso, E.; Pallàs, M.; Vázquez-Carrera, M. GADD45A: With or without you. Med. Res. Rev. 2024, 44, 1375–1403. [CrossRef] [PubMed]
35. Hoffman, B.; Liebermann, D.A. Gadd45 modulation of intrinsic and extrinsic stress responses in myeloid cells. J. Cell Physiol. 2009, 218, 26–31. [CrossRef]
36. Fresno Vara, J.A.; Casado, E.; de Castro, J.; Cejas, P.; Belda-Iniesta, C.; González-Barón, M. PI3K/Akt signalling pathway and cancer. Cancer Treat. Rev. 2004, 30, 193–204. [CrossRef]
37. Jafari, M.; Ghadami, E.; Dadkhah, T.; Akhavan-Niaki, H. PI3k/AKT signaling pathway: Erythropoiesis and beyond. J. Cell Physiol. 2019, 234, 2373–2385. [CrossRef]
38. Horta, C.A.; Doan, K.; Yang, J. Mechanotransduction pathways in regulating epithelial-mesenchymal plasticity. Curr. Opin. Cell Biol. 2023, 85, 102245. [CrossRef]
39. Levi, N.; Papismadov, N.; Solomonov, I.; Sagi, I.; Krizhanovsky, V. The ECM path of senescence in aging: Components and modifiers. FEBS J. 2020, 287, 2636–2646. [CrossRef]
40. Rosso, F.; Giordano, A.; Barbarisi, M.; Barbarisi, A. From cell-ECM interactions to tissue engineering. J. Cell Physiol. 2004, 199, 174–180. [CrossRef]
41. Jung, S.J.; Kim, H. Emerging View on the Molecular Functions of Sec62 and Sec63 in Protein Translocation. Int. J. Mol. Sci. 2021, 22, 12757. [CrossRef] [PubMed]
42. Colombo, S.F.; Fasana, E. Mechanisms of insertion of tail-anchored proteins into the membrane of the endoplasmic reticulum. Curr. Protein Pept. Sci. 2011, 12, 736–742. [CrossRef] [PubMed]
43. Mehlhorn, D.G.; Asseck, L.Y.; Grefen, C. Looking for a safe haven: Tail-anchored proteins and their membrane insertion pathways. Plant Physiol. 2021, 187, 1916–1928. [CrossRef]
44. Zhou, Y.; Wang, W.; Todorov, P.; Pei, C.; Isachenko, E.; Rahimi, G.; Mallmann, P.; Nawroth, F.; Isachenko, V. RNA Transcripts in Human Ovarian Cells: Two-Time Cryopreservation Does Not Affect Developmental Potential. Int. J. Mol. Sci. 2023, 24, 6880. [CrossRef]
45. Isachenko, V.; Morgenstern, B.; Todorov, P.; Isachenko, E.; Mallmann, P.; Hanstein, B.; Rahimi, G. Patient with ovarian insufficiency: Baby born after anticancer therapy and re-transplantation of cryopreserved ovarian tissue. J. Ovarian Res. 2020, 13, 118. [CrossRef]
46. Isachenko, V.; Mallmann, P.; Petrunkina, A.M.; Rahimi, G.; Nawroth, F.; Hancke, K.; Felberbaum, R.; Genze, F.; Damjanoski, I.; Isachenko, E. Comparison of in vitro- and chorioallantoic membrane (CAM)-culture systems for cryopreserved medulla-contained human ovarian tissue. PLoS ONE 2012, 7, e32549. [CrossRef]
47. Isachenko, V.; Todorov, P.; Isachenko, E.; Rahimi, G.; Hanstein, B.; Salama, M.; Mallmann, P.; Tchorbanov, A.; Hardiman, P.; Getreu, N.; et al. Cryopreservation and xenografting of human ovarian fragments: Medulla decreases the phosphatidylserine translocation rate. Reprod. Biol. Endocrinol. RBE 2016, 14, 79. [CrossRef]

4.8. APPENDIX

Figure

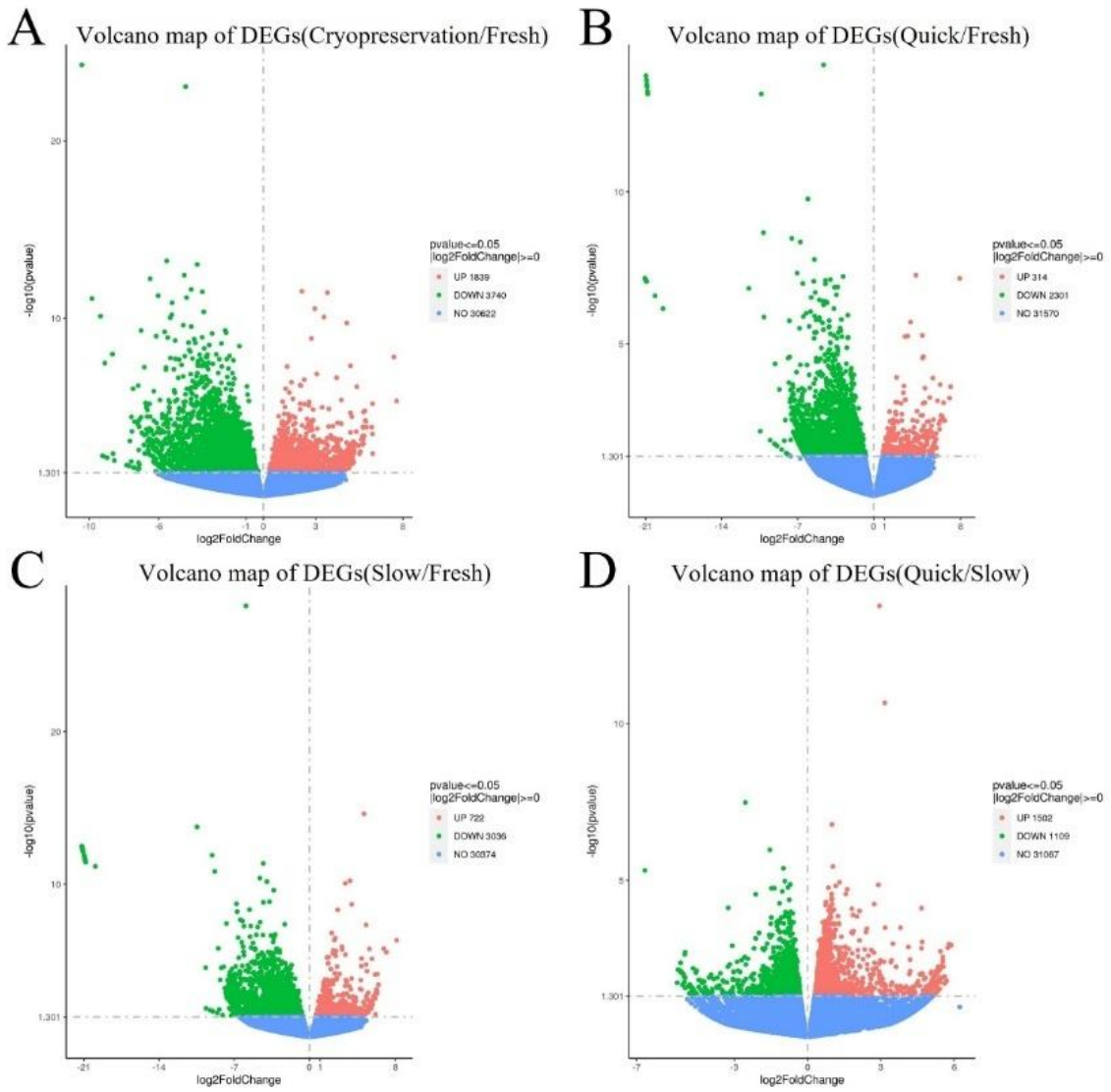


Figure 1. Volcano map of differentially expressed genes (DEGs) (A) DEGs volcano map: Group 2 plus Group 3 (cryopreserved ovarian tissue) vs. Group 1 (fresh ovarian tissue). (B) DEGs volcano map: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 1 (fresh ovarian tissue). (C) DEGs volcano map: Group 3 (frozen ovarian tissue after slow thawing at 37 °C) vs. Group 1 (fresh ovarian tissue). (D) DEGs volcano map: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 3 (frozen ovarian tissue after slow thawing at 37 °C).

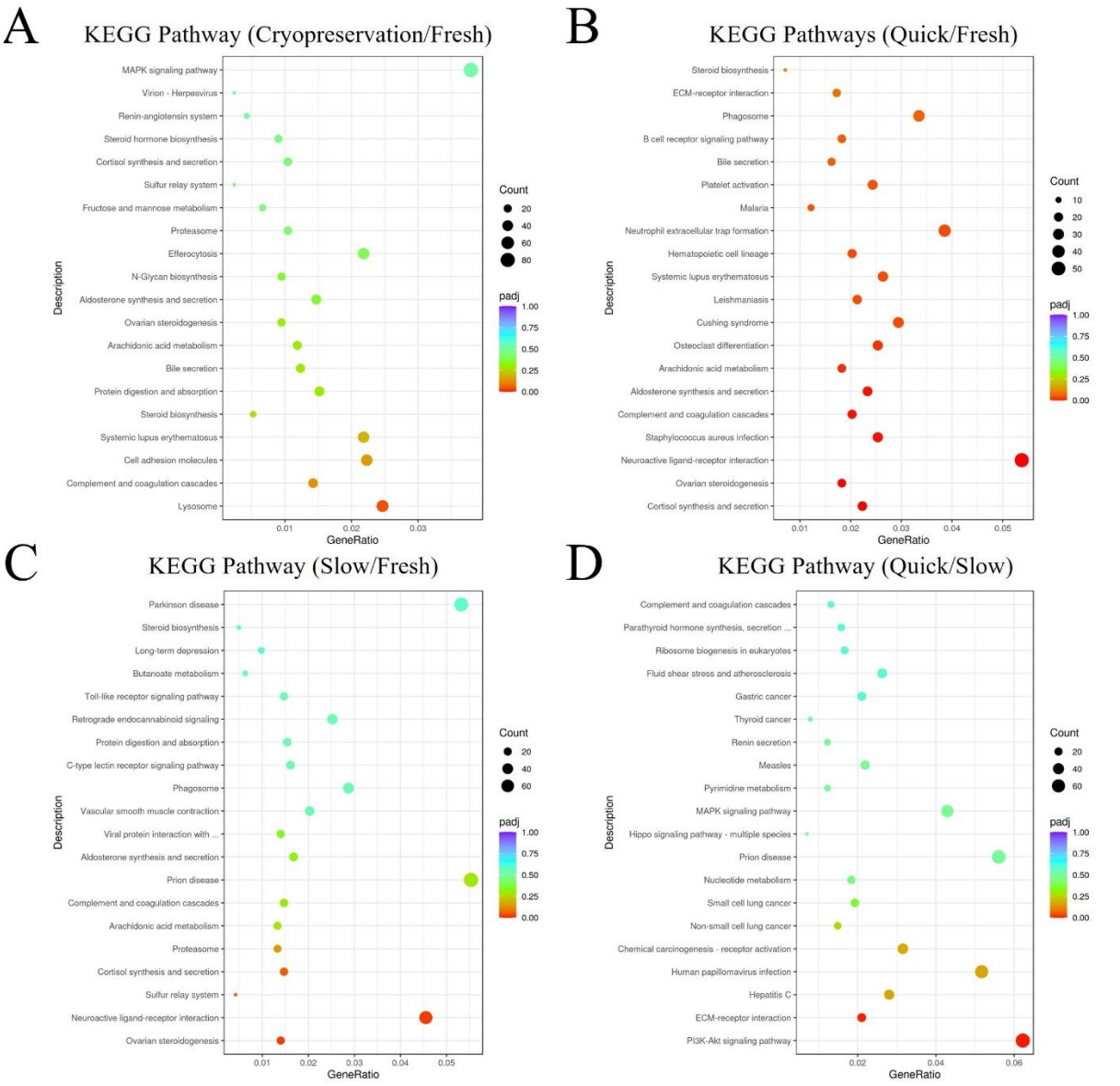
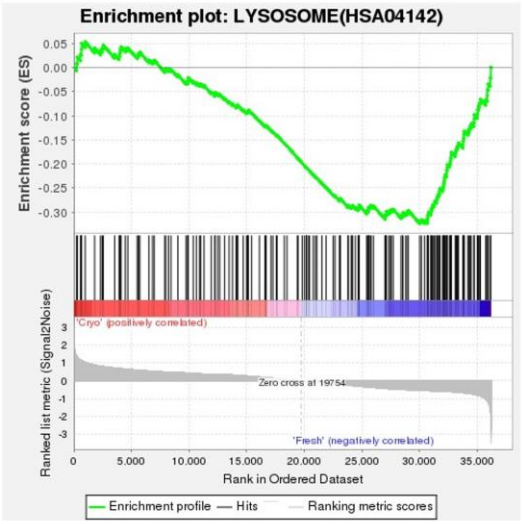


Figure 2. Visualization dot map of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (A) KEGG pathway analysis: Group 2 plus Group 3 (cryopreserved ovarian tissue) vs. Group 1 (fresh ovarian tissue). (B) KEGG pathway analysis: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 1 (fresh ovarian tissue). (C) KEGG pathway analysis: Group 3 (frozen ovarian tissue after slow thawing at 37 °C) vs. Group 1 (fresh ovarian tissue). (D) KEGG pathway analysis: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 3 (frozen ovarian tissue after slow thawing at 37 °C).

A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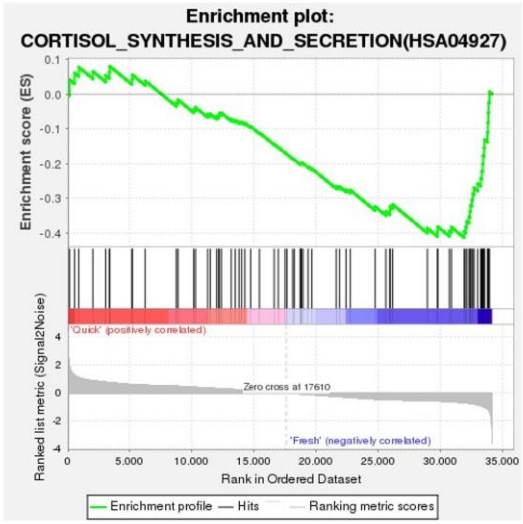
Zero cross at 19754
'Fresh' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

B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CORTISOL_SYNTHESIS_AND_SECRETION(HSA04927)
Enrichment score (ES)
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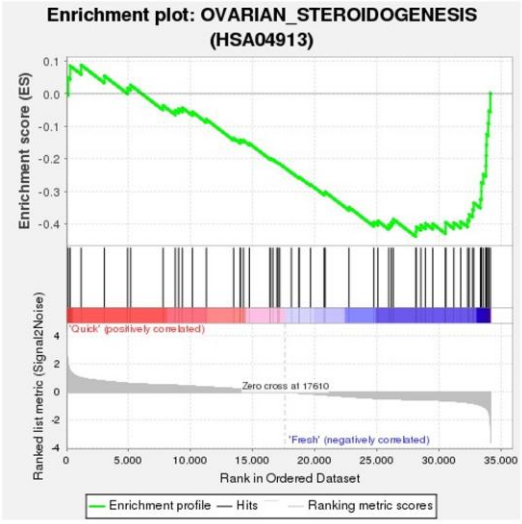
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

C


Enrichment plot: OVARIAN_STEROIDOGENESIS
(HSA04913)
Enrichment score (ES)
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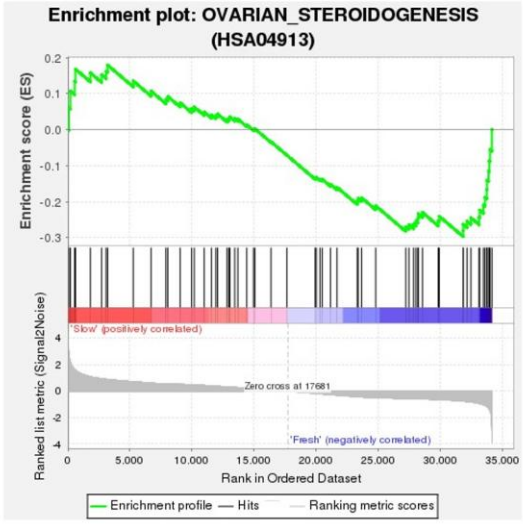
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

D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(HSA04913)
Enrichment score (ES)
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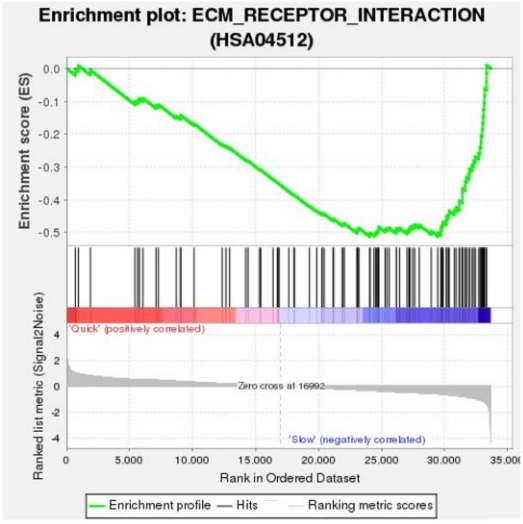
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

E


Enrichment plot: ECM_RECEPTOR_INTERACTION
(HSA04512)
Enrichment score (ES)
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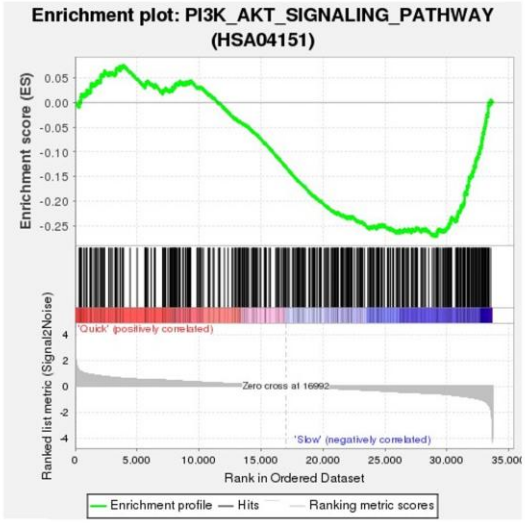
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

F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Zero cross at 16992
'Slow' (negatively correlated)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure 3. Gene Set Enrichment Analysis (GSEA) based on KEGG pathway data. (A) GSEA analysis indicated the lysosome pathway was enriched and downregulated in Group 2 plus Group 3 (cryopreserved ovarian tissue) compared with Group 1 (fresh ovarian tissue). (B) GSEA analysis indicated the cortisol synthesis pathway was enriched and downregulated in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 1 (fresh ovarian tissue). (C) GSEA analysis indicated the ovarian steroidogenesis pathway was enriched and downregulated in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 1 (fresh ovarian tissue). (D) GSEA analysis indicated the ovarian steroidogenesis pathway was enriched and downregulated in Group 3 (frozen ovarian tissue after slow thawing at 37 °C) vs. Group 1 (fresh ovarian tissue). (E) GSEA analysis indicated the PI3K-Akt signaling pathway was enriched and downregulated in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 3 (frozen ovarian tissue after slow thawing at 37 °C). (F) GSEA analysis indicated the ECM-receptor interaction pathway was enriched and downregulated in Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 3 (frozen ovarian tissue after slow thawing at 37 °C).

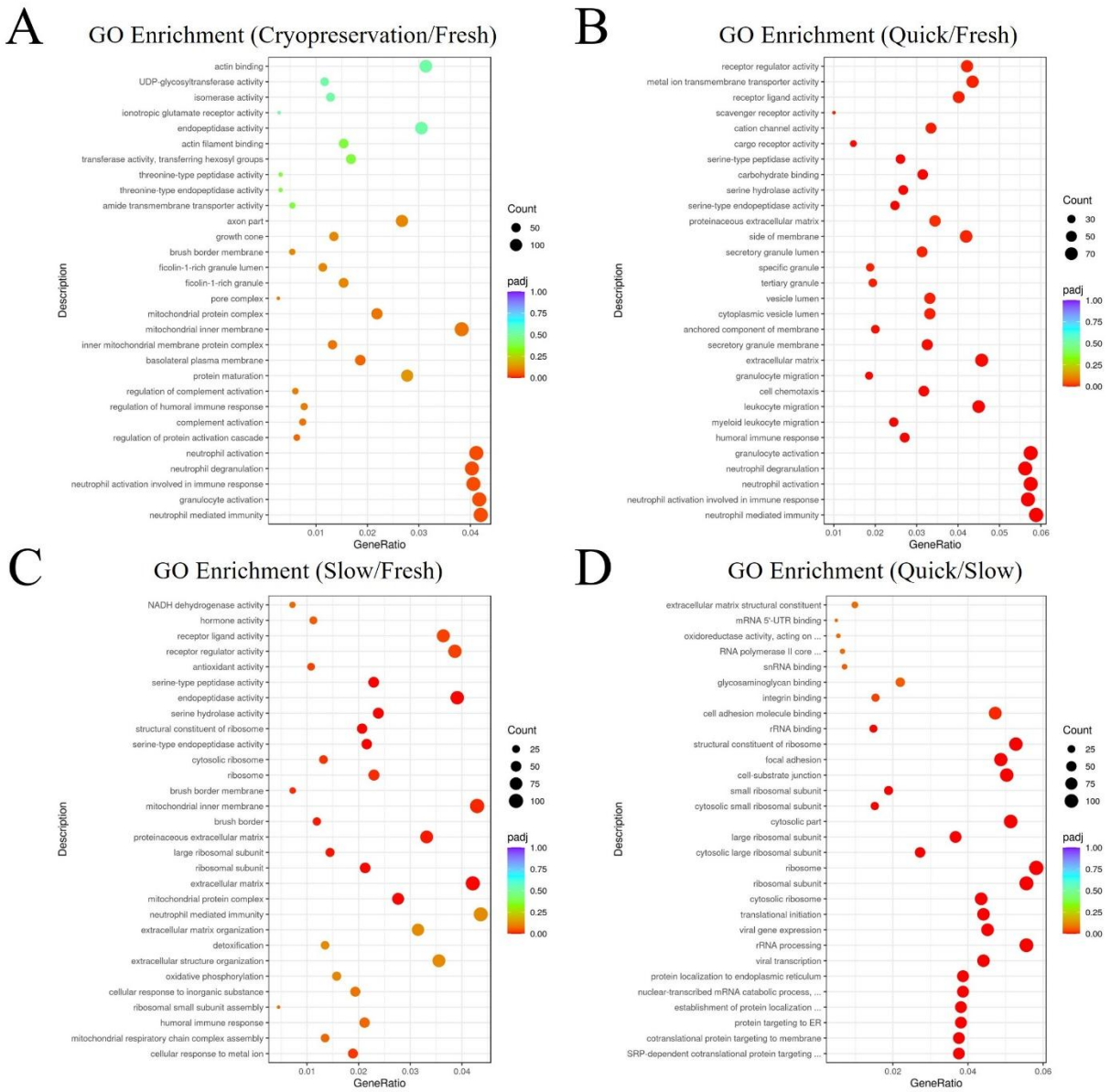

Figure 4. Visualization dot map of Gene Ontology (GO) enrichment analysis. (A) GO enrichment analysis: Group 2 plus Group 3 (cryopreserved ovarian tissue) vs. Group 1 (fresh ovarian tissue). (B) GO enrichment analysis: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 1 (fresh ovarian tissue). (C) GO enrichment analysis: Group 3 (frozen ovarian tissue after slow thawing at 37 °C) vs. Group 1 (fresh ovarian tissue). (D) GO enrichment analysis: Group 2 (frozen ovarian tissue after quick thawing at 100 °C) vs. Group 3 (frozen ovarian tissue after slow thawing at 37 °C).

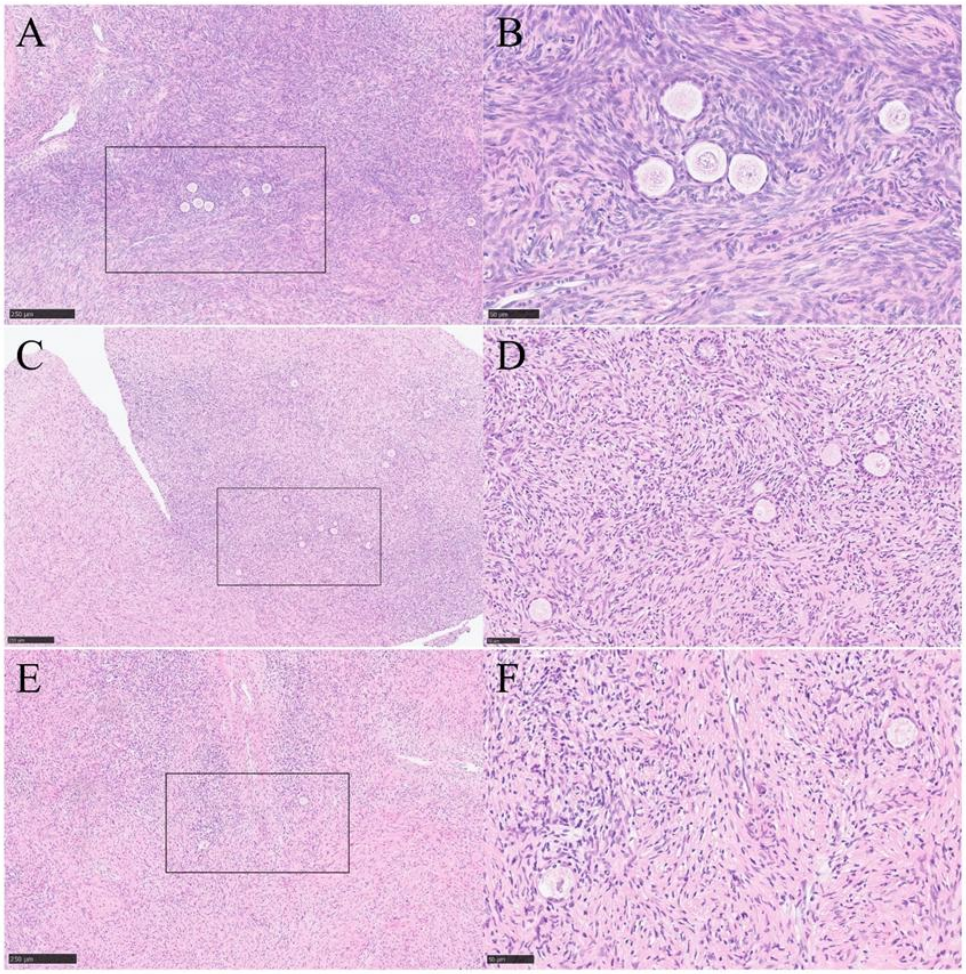

Figure 5. Hematoxylin-Eosin (HE) -staining of fresh ovarian tissue and cryopreserved ovarian tissue. (A,B) HE-staining of Group 1 (fresh ovarian tissue). (C,D) HE-staining of Group 2 (frozen ovarian tissue after quick thawing at 100 °C). (E,F) HE-staining of Group 3 Group 3 (frozen ovarian tissue after slow thawing at 37 °C).Bar for (A,C,E) = 250 µm, bar for (B,D,F) = 50 µm.

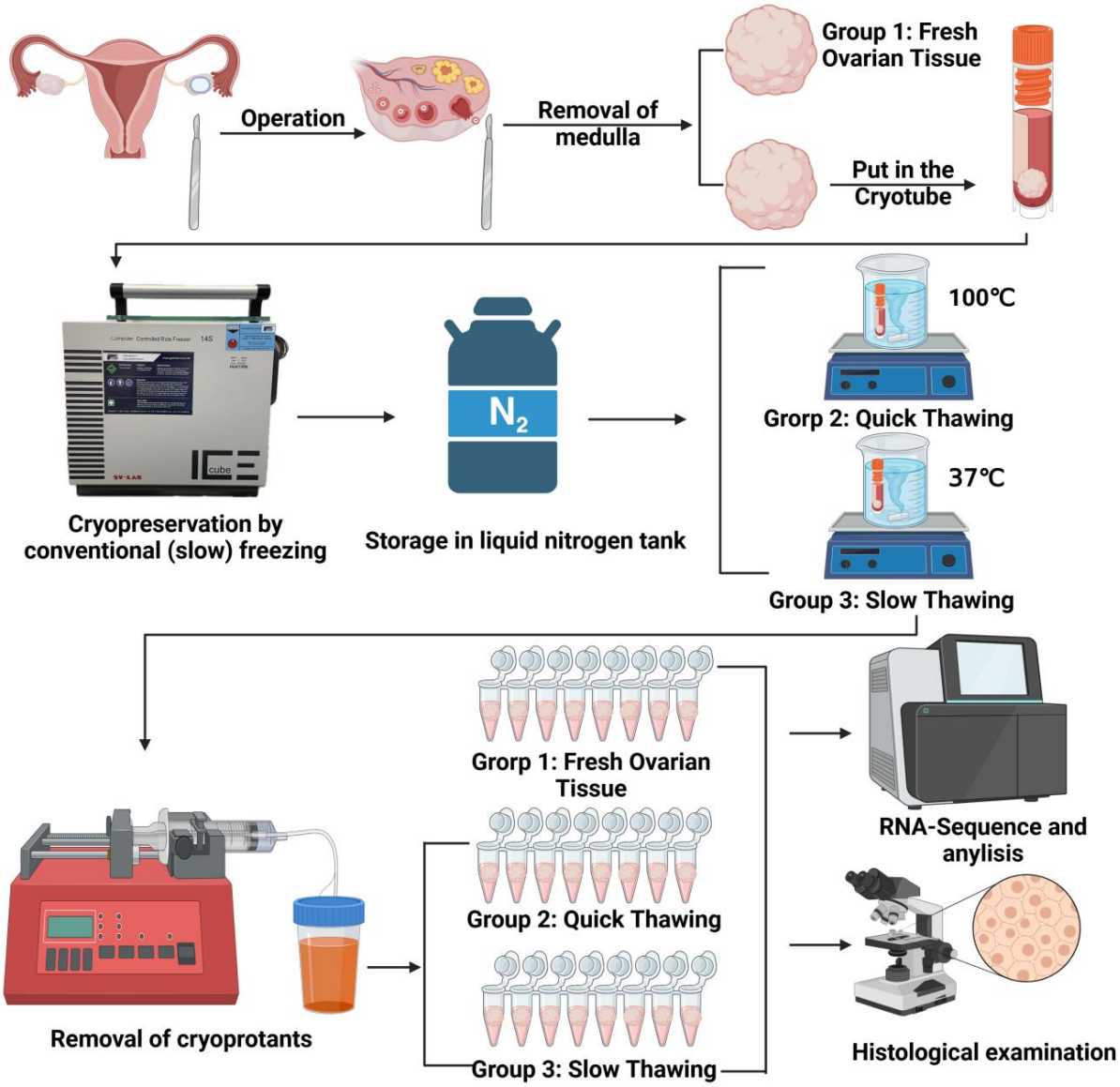

Figure 6. Design of experiments.

5. Discussion

Optimizing ovarian tissue cryopreservation technology is of great significance in the field of reproductive medicine and can better protect female fertility. During the freezing process, ovarian tissue is susceptible to various types of damage, including ice crystal formation, cell membrane rupture, and osmotic pressure imbalances. These factors lead to a decrease in follicle survival rates and structural damage to the ovarian matrix[30,69]. By optimizing the cryopreservation method, the survival and function of follicles and stromal cells can be significantly improved, laying the foundation for the reconstruction of ovarian function after subsequent transplantation. Therefore, efficient and controllable cryopreservation strategies have become the core prerequisite for future applications. In addition, optimizing freezing technology can better adapt to different patient groups and disease backgrounds and expand the scope of application of fertility preservation.

Based on the current strategies for preserving female fertility and the development of modern assisted reproductive technology, we first systematically reviewed and meta-analyzed the two mainstream cryopreservation methods, traditional slow freezing and vitrification. We found that the data included in the analysis showed that there was no significant difference between the two methods in the two main outcomes of follicle vitality and the proportion of intact primordial follicles. However, since the specific methods used in the included studies are not consistent, the results may be biased.

First, there are significant differences in the types, concentrations, and compositions of CPAs, which are one of the important variables affecting the cryopreservation effect of ovarian tissue. Different types of cryoprotectants vary in cell protection mechanisms, permeability, toxicity, and interactions with tissues, making it difficult to establish a unified standardized comparison system. For example, S. Silber et al. used human serum albumin (HSA) as an auxiliary protective agent in their study[74]. HSA has a good membrane-stabilizing effect and can interact with the cell membrane surface to reduce mechanical stress and ice crystal formation during freezing, thereby effectively reducing cell structural damage. However, in other studies, this component was not used, resulting in limited comparability between different experimental results. Similarly, A. Dalman et al. introduced catalase as an antioxidant component in their study [75]. Catalase can decompose the excessive ROS generated during freezing and thawing, thereby alleviating oxidative stress damage and protecting cell mitochondrial function and membrane integrity. This type of auxiliary cryoprotectant, based on antioxidant mechanisms, exhibits distinct protective advantages compared to traditional permeable CPAs.

Overall, the selection of cryoprotectants across studies lacks a unified standard. Not only do different types of cryoprotectants exist, but there is also considerable variability in concentration, exposure time, and combination ratios. While this diversity reflects the exploratory and adaptable nature of cryopreservation technology, it also increases heterogeneity in results, limiting cross-study comparisons and clinical application. Therefore, establishing a standardized system for cryoprotectant screening and application remains a key direction for future research. Systematic experiments should be conducted to evaluate the comprehensive effects of different CPAs and their combinations on ovarian tissue structure preservation, follicle survival, endocrine function recovery, and post-transplant fertility potential. This will further clarify the optimal formulation and operating conditions, thereby providing a unified and reproducible technical foundation for ovarian tissue cryopreservation.

Secondly, the size of the ovarian tissue specimen has a significant impact on the cryopreservation effect. During the freezing process of ovarian tissue, the thickness and volume of the tissue not only determine the penetration efficiency of the CPAs but also directly affect the uniformity of the cooling and thawing rates. Especially in vitrification, since it relies on an extremely high freezing rate to achieve amorphous solidification, if the tissue block is too large, it will be difficult to achieve uniform cooling inside and outside, resulting in delayed cooling in the central area of the tissue, residual ice crystals, or insufficient penetration of the protective agent. This temperature and osmotic difference can cause cell membrane rupture, mitochondrial damage, and local tissue necrosis, seriously affecting the survival rate of follicles and functional recovery after thawing. As pointed out by S. Zhao et al., the poor effect of vitrification of ovarian tissue is partly due to the large size of the tissue block[76]. The large tissue prevents the protective agent from fully penetrating the core of the tissue, causing cell frostbite and structural damage. According to this, traditional slow freezing offers a more controllable process, allowing for gradual equilibration of intracellular and extracellular water content during the gradual freezing process, thereby reducing osmotic stress and mechanical damage.

According to the systematic review and meta-analysis, this study chose to utilize the traditional slow freezing method for ovarian tissue cryopreservation. And the cryoprotectant solution chosen consisted of 6% DMSO, 6% EG, and 0.15 mol/L sucrose in a basal culture medium. DMSO and EG are typical osmotic CPAs, capable of entering cells to displace water and prevent ice crystal formation. Sucrose, a non-osmotic CPA, regulates extracellular osmotic pressure, aids dehydration, and mitigates osmotic cracking during rewarming. To ensure effective penetration of the cryoprotectant and uniform cooling, ovarian tissue specimens were standardized and cut into 0.5-1 cm^2 slices, approximately 1-2 mm thick. This specification ensures uniform diffusion of the cryoprotectant throughout the tissue while avoiding

temperature gradients caused by excessive volume, helping to maximize the integrity of the follicular structure and stromal cells.

In conclusion, according to the systematic review and meta-analysis, the optimization of subsequent experimental procedures has been done. By selecting the better cryopreservation method, optimizing the size of ovarian tissue, the type and concentration of cryoprotectants, tissue viability, and functional recovery rates after freezing and thawing can be significantly improved. These optimizations may provide a foundation for the standardization and broader clinical application of the ovarian tissue cryopreservation techniques.

This study also employed transcriptomics to compare cryopreserved ovarian tissues with fresh ovarian tissues. KEGG enrichment analysis revealed significant alterations in the pathways of cortisol synthesis and secretion, as well as ovarian steroidogenesis, between cryopreserved and fresh ovarian tissues. Further validation through GSEA confirmed the downregulation of both pathways. These findings indicate that, compared to fresh ovarian tissues, the pathways responsible for synthesizing estrogen and progesterone in cryopreserved ovarian tissues are notably downregulated, suggesting a reduction in the synthesis of these hormones. The development and function of ovarian tissue in vivo are intricately regulated by the hypothalamic-pituitary-ovarian (HPO) axis[77]. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which acts on the pituitary gland through the portal system, stimulating the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH mainly promotes the growth and development of follicles, while stimulating the granulosa cells of the follicles to secrete estrogen; LH plays a key role in the ovulation process, while stimulating the luteinization of the follicles and promoting the synthesis of progesterone. The estrogen and progesterone secreted by the ovaries regulate the secretion of GnRH, FSH and LH through feedback regulation of the hypothalamus and pituitary gland, thereby maintaining the dynamic balance of the entire reproductive endocrine system[78-81].

Therefore, the transcriptomic results of this study suggest that cryopreservation may temporarily inhibit the steroid hormone synthesis capacity of ovarian tissue, which may not only affect the maturation and ovulation function of follicles after thawing, but also interfere with the feedback regulation of the HPO axis, leading to delayed recovery of reproductive endocrine system function. This finding suggests that in the clinical application of frozen ovarian tissue, in addition to focusing on follicle survival and structural integrity, the hormone synthesis capacity and endocrine function recovery of the tissue after thawing should also be considered.

Under the control of the HPO axis, estrogen and progesterone regulate the secretion of GnRH, FSH, and LH through negative feedback, maintaining a dynamic balance between follicular growth, ovulation, and corpus luteum production[82,83]. Cryopreservation-induced downregulation of the steroidogenic pathway may temporarily impair this feedback loop, leading to abnormal FSH and LH levels and, in turn, affecting the synchronous development of follicles and ovulation efficiency in thawed ovarian tissue. Furthermore, hypothermia and freezing stress may induce oxidative stress and mitochondrial damage within follicular cells, further reducing follicular developmental potential. Despite this, experimental and clinical data indicate that most frozen ovarian tissue gradually recovers endocrine function and follicular activity after thawing, suggesting that this suppression may be temporary [27,84,85]. However, further optimization of cryopreservation strategies is needed to improve the physiological function and fertility recovery rate of thawed ovarian tissue. Adjustments to the cryoprotectant combination may be made to reduce oxidative stress, and supplemental gonadotropin stimulation can be used during transplantation or in vitro culture to promote rapid recovery of the steroidogenic pathway in thawed ovarian tissue. For instance, the inclusion of FSH and LH in the cryopreservation medium could promote the synthesis of estrogen and progesterone, thereby supporting the maintenance of ovarian reproductive endocrine function.

Despite advances in cryopreservation and thawing techniques, ovarian tissue still experiences varying degrees of damage during the freezing and thawing process, including injury to follicles, blood vessels, and stromal cells, which can compromise functional integrity. In vitro culture of ovarian tissue following cryopreservation has demonstrated potential for enhancing follicular viability and promoting tissue recovery, underscoring its importance in optimizing fertility outcomes[85].

To enhance the survival rate of ovarian tissue transplantation, in vitro culture of cryopreserved ovarian tissue has become a promising strategy[86,87]. Various in vitro culture strategies have been explored, including two-dimensional (2D) culture, three-dimensional (3D) suspension culture, microfluidic culture, and 3D scaffold-based culture. In 2D culture, ovarian tissue fragments are placed on a culture dish with medium; however, the absence of a 3D structural environment limits follicular development. In contrast, 3D suspension culture preserves tissue architecture and improves follicular survival and maturation by preventing adhesion, although maintaining structural stability remains challenging. Microfluidic culture, which utilizes microfluidic chips to precisely control nutrient supply, is well-suited for long-term culture but is limited by technical complexity and high cost. Finally, 3D scaffold-based culture involves embedding ovarian tissue within natural or synthetic biomaterials, facilitating cell–cell interactions, enhancing follicular viability, and advancing artificial ovary development. The

selection of appropriate biomaterials is critical, as they significantly influence tissue functionality and developmental outcomes[88-90].

Recent studies indicated that a 3D culture model using TISSEEL fibrin, designed to simulate the native stromal architecture. This 3D system provides stable structural support, preserving the natural spatial organization of tissue and thereby improving tissue survival. The TISSEEL fibrin 3D scaffold effectively promoted follicle development; however, it also increased the metabolic activity within the tissue, leading to some degree of cellular damage. Elevated metabolic demands combined with limited oxygen diffusion are common challenges in tissue culture. The addition of antioxidants may help mitigate oxidative stress and reduce cell injury. Furthermore, microfluidic systems, which can enhance the uniform distribution of oxygen and nutrients and prevent central hypoxia and necrosis, represent a potential improvement, although their technical complexity currently limits widespread application[91-93]. Future investigations in this area are highly encouraged.

While the TISSEEL fibrin 3D scaffold provides crucial spatial support and promotes intercellular communication, prolonged culture leads to weakened cell adhesion, potentially due to gradual fibrin degradation. Additionally, extended culture times can result in insufficient nutrient and energy supply, ultimately causing cell necrosis. Therefore, optimizing the in vitro culture duration is critical, requiring a balance between supporting follicular development and preventing tissue necrosis or functional decline associated with prolonged culture.

This study aimed to optimize ovarian tissue cryopreservation techniques to improve follicle survival and endocrine function recovery after freezing and thawing. Based on an analysis of current female fertility preservation strategies and modern assisted reproductive technologies, we first compared the effectiveness of different cryopreservation methods, including traditional slow freezing and vitrification. Taking into account tissue survival, operational controllability, and clinical feasibility, this study selected traditional slow freezing as the primary preservation strategy. Furthermore, to minimize ice crystal recrystallization and osmotic stress damage during thawing, we optimize the thawing rate during thawing.

During cryopreservation, the type, concentration, and composition of CPAs are crucial for tissue preservation. Permeable CPAs, such as DMSO and EG, can enter cells to displace water and reduce ice crystal formation, while non-permeable CPAs, such as sucrose, can assist in dehydration by regulating extracellular osmotic pressure, reducing the risk of rewarming and cracking. Furthermore, the combination of cryoprotectants and the gradient exposure time requires careful control to balance protective efficacy with toxicity. Research

has shown that the addition of supplements such as HSA or antioxidant enzymes, such as catalase, can further reduce ice crystal formation and oxidative stress damage, thereby improving follicle survival.

The size and thickness of ovarian tissue specimens are also important factors influencing freezing efficacy. To ensure adequate penetration of the cryoprotectant and uniform cooling, this study cut the ovarian cortex into slices 0.5–1 cm^2 and 1–2 mm thick. This size ensures rapid cooling within the tissue while maintaining structural integrity, maximizing the protection of follicles and stromal cells.

To further enhance tissue function after cryopreservation, the in vitro three-dimensional culture model to simulate the ovarian microenvironment and promote follicle growth and maturation is viable. Future efforts within this three-dimensional culture system could include the addition of FSH, LH, or antioxidants, as well as integrated microfluidic systems to optimize the exchange of nutrients and signaling molecules, thereby enhancing ovarian tissue functional recovery and fertility potential.

Overall, this study not only improved the survival rate and functional recovery of ovarian tissue by selecting an appropriate freezing method, optimizing cryoprotectant formulations, and thawing conditions, but also provided clear direction for further improving freezing-thawing protocols. Future studies could further systematically evaluate the optimal conditions for hormone and antioxidant combinations, and explore the application of advanced technologies such as microfluidics in ovarian tissue cryopreservation to maximize structural protection and functional recovery.

6. References

1. Oktay K, Harvey BE, Partridge AH, et al. Fertility Preservation in Patients With Cancer: ASCO Clinical Practice Guideline Update. *J Clin Oncol* 2018; **36**(19): 1994-2001.
2. Cobo A, García-Velasco JA, Remohí J, Pellicer A. Oocyte vitrification for fertility preservation for both medical and nonmedical reasons. *Fertility and sterility* 2021; **115**(5): 1091-101.
3. Gook DA, Edgar DH. Human oocyte cryopreservation. *Human reproduction update* 2007; **13**(6): 591-605.
4. Liang T, Motan T. Mature Oocyte Cryopreservation for Fertility Preservation. *Advances in experimental medicine and biology* 2016; **951**: 155-61.
5. Jain JK, Paulson RJ. Oocyte cryopreservation. *Fertility and sterility* 2006; **86**(4 Suppl): 1037-46.
6. Clark NA, Swain JE. Oocyte cryopreservation: searching for novel improvement strategies. *Journal of assisted reproduction and genetics* 2013; **30**(7): 865-75.
7. Levi-Setti PE, Patrizio P, Scaravelli G. Evolution of human oocyte cryopreservation: slow freezing versus vitrification. *Curr Opin Endocrinol Diabetes Obes* 2016; **23**(6): 445-50.
8. Glujovsky D, Riestra B, Sueldo C, et al. Vitrification versus slow freezing for women undergoing oocyte cryopreservation. *Cochrane Database Syst Rev* 2014; **2014**(9): Cd010047.
9. Mature oocyte cryopreservation: a guideline. *Fertility and sterility* 2013; **99**(1): 37-43.
10. Doyle JO, Richter KS, Lim J, Stillman RJ, Graham JR, Tucker MJ. Successful elective and medically indicated oocyte vitrification and warming for autologous in vitro fertilization, with predicted birth probabilities for fertility preservation according to number of cryopreserved oocytes and age at retrieval. *Fertility and sterility* 2016; **105**(2): 459-66.e2.
11. Paynter SJ, Cooper A, Gregory L, Fuller BJ, Shaw RW. Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide. *Human reproduction (Oxford, England)* 1999; **14**(9): 2338-42.
12. Paynter SJ, Fuller BJ, Shaw RW. Temperature dependence of mature mouse oocyte membrane permeabilities in the presence of cryoprotectant. *Cryobiology* 1997; **34**(2): 122-30.
13. Niemann H, Lucas-Hahn A, Stoffregen C. Cryopreservation of bovine oocytes and embryos following microsurgical operations. *Molecular reproduction and development* 1993; **36**(2): 232-5.
14. Tao T, Del Valle A. Human oocyte and ovarian tissue cryopreservation and its application. *Journal of assisted reproduction and genetics* 2008; **25**(7): 287-96.
15. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and sterility* 1990; **54**(1): 102-8.
16. Rayos AA, Takahashi Y, Hishinuma M, Kanagawa H. Quick freezing of unfertilized mouse oocytes using ethylene glycol with sucrose or trehalose. *J Reprod Fertil* 1994; **100**(1): 123-9.
17. Coticchio G, De Santis L, Rossi G, et al. Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Human reproduction (Oxford, England)* 2006; **21**(7): 1771-6.
18. Regmi R, Winkler PM, Flauraud V, et al. Planar Optical Nanoantennas Resolve Cholesterol-Dependent Nanoscale Heterogeneities in the Plasma Membrane of Living Cells. *Nano Lett* 2017; **17**(10): 6295-302.
19. Meintjes M, Chantilis SJ, Ward DC, et al. A randomized controlled study of human serum albumin and serum substitute supplement as protein supplements for IVF culture and the effect on live birth rates. *Human reproduction (Oxford, England)* 2009; **24**(4): 782-9.
20. Cao B, Qin J, Pan B, et al. Oxidative Stress and Oocyte Cryopreservation: Recent Advances in Mitigation Strategies Involving Antioxidants. *Cells* 2022; **11**(22).
21. Gualtieri R, Kalthur G, Barbato V, Di Nardo M, Adiga SK, Talevi R. Mitochondrial Dysfunction and Oxidative Stress Caused by Cryopreservation in Reproductive Cells. *Antioxidants (Basel)* 2021; **10**(3).
22. Keane JA, Ealy AD. An Overview of Reactive Oxygen Species Damage Occurring during In Vitro Bovine Oocyte and Embryo Development and the Efficacy of Antioxidant Use to Limit

These Adverse Effects. *Animals : an open access journal from MDPI* 2024; **14**(2).
23. Moawad AR, Tan SL, Taketo T. Beneficial effects of glutathione supplementation during vitrification of mouse oocytes at the germinal vesicle stage on their preimplantation development following maturation and fertilization in vitro. *Cryobiology* 2017; **76**: 98-103.
24. Olexiková L, Dujíčková L, Makarevich AV, et al. Glutathione during Post-Thaw Recovery Culture Can Mitigate Deleterious Impact of Vitrification on Bovine Oocytes. *Antioxidants (Basel)* 2022; **12**(1).
25. Pavone ME, Innes J, Hirshfeld-Cytron J, Kazer R, Zhang J. Comparing thaw survival, implantation and live birth rates from cryopreserved zygotes, embryos and blastocysts. *Journal of human reproductive sciences* 2011; **4**(1): 23-8.
26. Cascante SD, Berkeley AS, Licciardi F, McCaffrey C, Grifo JA. Planned oocyte cryopreservation: the state of the ART. *Reproductive biomedicine online* 2023; **47**(6): 103367.
27. Dolmans MM, von Wolff M, Poirot C, et al. Transplantation of cryopreserved ovarian tissue in a series of 285 women: a review of five leading European centers. *Fertility and sterility* 2021; **115**(5): 1102-15.
28. Dunlop CE, Anderson RA. Clinical dilemmas in ovarian tissue cryopreservation. *Fertility and sterility* 2024; **122**(4): 559-64.
29. Gadek LM, Joswiak C, Laronda MM. Thawing fertility: a view of ovarian tissue cryopreservation processes and review of ovarian transplant research. *Fertility and sterility* 2024; **122**(4): 574-85.
30. Fabbri R, Vicenti R, Magnani V, et al. Ovarian tissue cryopreservation and transplantation: 20 years experience in Bologna University. *Frontiers in endocrinology* 2022; **13**: 1035109.
31. Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. *Human reproduction update* 2006; **12**(5): 519-35.
32. Cacciottola L, Donnez J, Dolmans MM. Ovarian tissue and oocyte cryopreservation prior to iatrogenic premature ovarian insufficiency. *Best practice & research Clinical obstetrics & gynaecology* 2022; **81**: 119-33.
33. Fraison E, Huberlant S, Labrune E, et al. Live birth rate after female fertility preservation for cancer or haematopoietic stem cell transplantation: a systematic review and meta-analysis of the three main techniques; embryo, oocyte and ovarian tissue cryopreservation. *Human reproduction (Oxford, England)* 2023; **38**(3): 489-502.
34. Behl S, Joshi VB, Larson NB, et al. Vitrification versus slow freezing of human ovarian tissue: a systematic review and meta-analysis of histological outcomes. *Journal of assisted reproduction and genetics* 2023; **40**(3): 455-64.
35. Isachenko V, Isachenko E, Reinsberg J, et al. Cryopreservation of human ovarian tissue: comparison of rapid and conventional freezing. *Cryobiology* 2007; **55**(3): 261-8.
36. Jaeger P, Fournier C, Santamaria C, et al. Human ovarian cryopreservation: vitrification versus slow freezing from histology to gene expression. *Human fertility (Cambridge, England)* 2023; **26**(5): 1099-107.
37. Bahroudi Z, Zarnaghi MR, Izadpanah M, et al. Review of ovarian tissue cryopreservation techniques for fertility preservation. *Journal of gynecology obstetrics and human reproduction* 2022; **51**(2): 102290.
38. Amorim CA, Gonçalves PB, Figueiredo JR. Cryopreservation of oocytes from pre-antral follicles. *Human reproduction update* 2003; **9**(2): 119-29.
39. Bromer JG, Patrizio P. Fertility preservation: the rationale for cryopreservation of the whole ovary. *Seminars in reproductive medicine* 2009; **27**(6): 465-71.
40. Donnez J, Dolmans MM. Cryopreservation of ovarian tissue: an overview. *Minerva medica* 2009; **100**(5): 401-13.
41. Hoekman EJ, Louwe LA, Rooijers M, et al. Ovarian tissue cryopreservation: Low usage rates and high live-birth rate after transplantation. *Acta obstetricia et gynecologica Scandinavica* 2020; **99**(2): 213-21.
42. Fabbri R, Vicenti R, Magnani V, et al. Cryopreservation of ovarian tissue in breast cancer patients: 10 years of experience. *Future oncology (London, England)* 2012; **8**(12): 1613-9.
43. Donnez J, Squifflet J, Dolmans MM. Frozen-thawed ovarian tissue retransplants. *Seminars in reproductive medicine* 2009; **27**(6): 472-8.

44. Gudlevičienė Ž, Žilinskas K, Kundrotas G, Grubliauskaitė M, Baltriukienė D, Bukelskienė V. Slow-Freezing Cryopreservation Ensures High Ovarian Tissue Quality Followed by In Vivo and In Vitro Methods and Is Safe for Fertility Preservation. *Medicina (Kaunas)* 2020; **56**(10).
45. Hajati F, Kashi AM, Totonchi M, Valojerdi MR. Post-thawing and culture comparison of three routine slow freezing methods for human ovarian tissue cryopreservation: Histological, molecular, and hormonal aspects. *Cryobiology* 2022; **104**: 32-41.
46. Kometas M, Christman GM, Kramer J, Rhoton-Vlasak A. Methods of Ovarian Tissue Cryopreservation: Is Vitrification Superior to Slow Freezing?-Ovarian Tissue Freezing Methods. *Reproductive sciences (Thousand Oaks, Calif)* 2021; **28**(12): 3291-302.
47. Sanfilippo S, Canis M, Smitz J, et al. Vitrification of human ovarian tissue: a practical and relevant alternative to slow freezing. *Reproductive biology and endocrinology : RB&E* 2015; **13**: 67.
48. Shi Q, Xie Y, Wang Y, Li S. Vitrification versus slow freezing for human ovarian tissue cryopreservation: a systematic review and meta-anlaysis. *Scientific reports* 2017; **7**(1): 8538.
49. Elliott GD, Wang S, Fuller BJ. Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology* 2017; **76**: 74-91.
50. Rajan R, Matsumura K. Development and Application of Cryoprotectants. *Advances in experimental medicine and biology* 2018; **1081**: 339-54.
51. El Cury-Silva T, Nunes MEG, Casalechi M, Comim FV, Rodrigues JK, Reis FM. Cryoprotectant agents for ovarian tissue vitrification: Systematic review. *Cryobiology* 2021; **103**: 7-14.
52. Dougherty RM. Use of dimethyl sulphoxide for preservation of tissue culture cells by freezing. *Nature* 1962; **193**: 550-2.
53. Yu ZW, Quinn PJ. Dimethyl sulphoxide: a review of its applications in cell biology. *Biosci Rep* 1994; **14**(6): 259-81.
54. Gandolfi F, Paffoni A, Papasso Brambilla E, Bonetti S, Brevini TA, Ragni G. Efficiency of equilibrium cooling and vitrification procedures for the cryopreservation of ovarian tissue: comparative analysis between human and animal models. *Fertility and sterility* 2006; **85 Suppl 1**: 1150-6.
55. Huang L, Mo Y, Wang W, Li Y, Zhang Q, Yang D. Cryopreservation of human ovarian tissue by solid-surface vitrification. *European journal of obstetrics, gynecology, and reproductive biology* 2008; **139**(2): 193-8.
56. Isachenko E, Isachenko V, Rahimi G, Nawroth F. Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *European journal of obstetrics, gynecology, and reproductive biology* 2003; **108**(2): 186-93.
57. Rahimi G, Isachenko E, Sauer H, et al. Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. *Reproduction, fertility, and development* 2003; **15**(6): 343-9.
58. Tian T, Zhao G, Han D, et al. Effects of vitrification cryopreservation on follicular morphology and stress relaxation behaviors of human ovarian tissues: sucrose versus trehalose as the non-permeable protective agent. *Human reproduction (Oxford, England)* 2015; **30**(4): 877-83.
59. Cheung RCF, Ng TB, Wong JH. Antifreeze Proteins from Diverse Organisms and their Applications: An Overview. *Curr Protein Pept Sci* 2017; **18**(3): 262-83.
60. Ekpo MD, Xie J, Hu Y, et al. Antifreeze Proteins: Novel Applications and Navigation towards Their Clinical Application in Cryobanking. *International journal of molecular sciences* 2022; **23**(5).
61. Robles V, Valcarce DG, Riesco MF. The Use of Antifreeze Proteins in the Cryopreservation of Gametes and Embryos. *Biomolecules* 2019; **9**(5).
62. Wu X, Yao F, Zhang H, Li J. Antifreeze proteins and their biomimetics for cell cryopreservation: Mechanism, function and application-A review. *Int J Biol Macromol* 2021; **192**: 1276-91.
63. Takago S, Matsumoto I, Kato H, et al. Hypothermic preservation of rat hearts using antifreeze glycoprotein. *Physiol Res* 2020; **69**(6): 1029-38.
64. Kong HS, Kim EJ, Youm HW, et al. Improvement in Ovarian Tissue Quality with

Supplementation of Antifreeze Protein during Warming of Vitrified Mouse Ovarian Tissue. *Yonsei medical journal* 2018; **59**(2): 331-6.
65. Zeng Q, Wang K, He LB, Wang TT, Fan XM, Liu WX. Cryoprotective effect of antifreeze protein III on the rabbit ovary. *Reproduction, fertility, and development* 2022; **34**(9): 645-57.
66. Kong HS, Hong YH, Lee J, et al. Antifreeze Protein Supplementation During the Warming of Vitrified Bovine Ovarian Tissue Can Improve the Ovarian Tissue Quality After Xenotransplantation. *Frontiers in endocrinology* 2021; **12**: 672619.
67. Leonel ECR, Corral A, Risco R, et al. Stepped vitrification technique for human ovarian tissue cryopreservation. *Scientific reports* 2019; **9**(1): 20008.
68. Isachenko V, Isachenko E, Rahimi G, Krivokharchenko A, Alabart JL, Nawroth F. Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen: negative effect of disaccharides in vitrification solution. *Cryo letters* 2002; **23**(5): 333-44.
69. Isachenko V, Dittrich R, Keck G, et al. Cryopreservation of Ovarian Tissue: Detailed Description of Methods for Transport, Freezing and Thawing. *Geburtshilfe Frauenheilkd* 2012; **72**(10): 927-32.
70. Isachenko V, Morgenstern B, Todorov P, et al. Patient with ovarian insufficiency: baby born after anticancer therapy and re-transplantation of cryopreserved ovarian tissue. *Journal of ovarian research* 2020; **13**(1): 118.
71. Zhou Y, Wang W, Todorov P, et al. RNA Transcripts in Human Ovarian Cells: Two-Time Cryopreservation Does Not Affect Developmental Potential. *International journal of molecular sciences* 2023; **24**(8).
72. Chavoshinezhad N, Niknafs B. Innovations in 3D ovarian and follicle engineering for fertility preservation and restoration. *Molecular biology reports* 2024; **51**(1): 1004.
73. Schallmoser A, Emrich N, Einenkel R, Sänger N. Explorative 3-D culture of early secondary follicles in a time lapse system for up to 36 days gives valuable, but limited insights in follicular development. *Placenta* 2025; **164**: 50-63.
74. Silber S, Kagawa N, Kuwayama M, Gosden R. Duration of fertility after fresh and frozen ovary transplantation. *Fertility and sterility* 2010; **94**(6): 2191-6.
75. Dalman A, Deheshkar Gooneh Farahani NS, Totonchi M, Pirjani R, Ebrahimi B, Rezazadeh Valojerdi M. Slow freezing versus vitrification technique for human ovarian tissue cryopreservation: An evaluation of histological changes, WNT signaling pathway and apoptotic genes expression. *Cryobiology* 2017; **79**: 29-36.
76. Zhao S, Zhao SQ, Shi Q, et al. Effects of different freezing methods on the follicular viability of large pieces of human ovarian tissues. *Acta Anatomica Sinica* 2013; **44**(3): 427-32.
77. Apter D. Development of the hypothalamic-pituitary-ovarian axis. *Ann N Y Acad Sci* 1997; **816**: 9-21.
78. Mahesh VB, Murphy LL, O'Conner JL. Selective modulation of FSH and LH secretion by steroids. *Advances in experimental medicine and biology* 1987; **219**: 131-52.
79. Kaprara A, Huhtaniemi IT. The hypothalamus-pituitary-gonad axis: Tales of mice and men. *Metabolism* 2018; **86**: 3-17.
80. Stamatiades GA, Kaiser UB. Gonadotropin regulation by pulsatile GnRH: Signaling and gene expression. *Mol Cell Endocrinol* 2018; **463**: 131-41.
81. Charlesworth MC, Schwartz NB. Estrogen inhibition of LH and FSH secretion: effects of a GnRH antagonist. *Am J Physiol* 1986; **250**(4 Pt 1): E341-5.
82. Mikhael S, Punjala-Patel A, Gavrilova-Jordan L. Hypothalamic-Pituitary-Ovarian Axis Disorders Impacting Female Fertility. *Biomedicines* 2019; **7**(1).
83. Buttram VC, Jr. The immature HPO axis. *The Journal of reproductive medicine* 1975; **14**(1): 21-5.
84. Finkelstein T, Rolnik DL, Agresta F, et al. Pregnancy outcomes following ovarian tissue cryopreservation: an Australian cohort study. *American journal of obstetrics and gynecology* 2025; **233**(2): 108.e1-.e11.
85. Jadoul P, Guilmain A, Squifflet J, et al. Efficacy of ovarian tissue cryopreservation for fertility preservation: lessons learned from 545 cases. *Human reproduction (Oxford, England)* 2017; **32**(5): 1046-54.
86. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FD. 3D cell culture systems: advantages and applications. *J Cell Physiol* 2015; **230**(1): 16-26.

87. Corrò C, Novellasdemunt L, Li VSW. A brief history of organoids. *Am J Physiol Cell Physiol* 2020; **319**(1): C151-c65.
88. Khunmanee S, Park H. Three-Dimensional Culture for In Vitro Folliculogenesis in the Aspect of Methods and Materials. *Tissue Eng Part B Rev* 2022; **28**(6): 1242-57.
89. Brito IR, Lima IM, Xu M, Shea LD, Woodruff TK, Figueiredo JR. Three-dimensional systems for in vitro follicular culture: overview of alginate-based matrices. *Reproduction, fertility, and development* 2014; **26**(7): 915-30.
90. Kim EJ, Yang C, Lee J, et al. The new biocompatible material for mouse ovarian follicle development in three-dimensional in vitro culture systems. *Theriogenology* 2020; **144**: 33-40.
91. Mehling M, Tay S. Microfluidic cell culture. *Curr Opin Biotechnol* 2014; **25**: 95-102.
92. Kuzmic N, Moore T, Devadas D, Young EWK. Modelling of endothelial cell migration and angiogenesis in microfluidic cell culture systems. *Biomech Model Mechanobiol* 2019; **18**(3): 717-31.
93. Li XJ, Valadez AV, Zuo P, Nie Z. Microfluidic 3D cell culture: potential application for tissue-based bioassays. *Bioanalysis* 2012; **4**(12): 1509-25.