Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe der Universität zu Köln
Direktor: Universitätsprofessor Dr. med. P. K. Mallmann

RNA sequencing to explore the transcriptome differences of testicular tissues at different stages of cryopreservation under different thawing methods and in vitro culture

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
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promoviert am 21. Oktober 2025

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln

Druckjahr 2025

Dekan: Universitätsprofessor Dr. med. G. R. Fink

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Diese Daten wurden von Mengyang Cao, Qingduo Kong, Evgenia Isachenko, Gohar Rahimi, Nina Mallmann-Gottschalk, Volodimir Isachenko und von mir zusammen ausgewertet.

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Danksagung

First and foremost, I am deeply grateful to Professor Volodimir Isachenko for his unwavering support and guidance since I arrived in Germany. From the very beginning, he encouraged me to explore every new idea, no matter how unconventional it seemed. His belief in my abilities, combined with his insightful advice, empowered me to push the boundaries of my research. During moments of doubt and confusion, Professor Isachenko was always there with patience and selfless help, offering both intellectual and emotional support. His mentorship has been invaluable, and without his encouragement, this project would not have reached completion.

I would also like to extend my heartfelt thanks to all of my colleagues in our research group. Your collaboration, feedback, and camaraderie made this journey both rewarding and enjoyable. The stimulating discussions, shared experiences, and collective problem-solving were crucial in shaping my work. I feel fortunate to have been part of such a dynamic and supportive team.

Finally, I would like to give special thanks to my beloved parents for giving me life and supporting me to pursue my ideals and ambitions.

Widmung

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DEG	Differentially Expressed Genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein–Protein Interactions

1. Zusammenfassung

Es wurden keine wesentlichen Unterschiede in der Qualität der Zellen reifen und unreifen Hodengewebes nach der Kryokonservierung festgestellt. Generell war das Auftauen von reifem und unreifem Hodengewebe bei 100 °C effektiver. Der größte Unterschied in der Intensität der Genexpression wurde bei Ribosomen von bei 100 °C aufgetauten Zellen im Vergleich zu bei 37 °C aufgetauten Zellen beobachtet. Zusammenfassend lässt sich sagen, dass eine höhere Auftaugeschwindigkeit für gefrorenes Hodengewebe von Vorteil ist.

Die Verkapselung von Hodengewebe in Fibrin und die Langzeit-In-vitro-Kultur unter ständigem Rühren in einem großen Volumen Kulturmedium können die Auswirkungen der Auftaumethoden auf kryokonserviertes Hodengewebe reduzieren.

2. Introduction

Cryopreservation of semen has long been established as the standard approach for preserving male fertility. Semen retrieval can be achieved through various techniques, including direct ejaculation, testicular sperm aspiration (TESA), testicular sperm extraction (TESE), percutaneous epididymal sperm aspiration (PESA), and microsurgical epididymal sperm aspiration (MESA)[1]. TESA entails the percutaneous insertion of a needle into testicular tissue to aspirate sperm, serving as a minimally invasive technique primarily employed for obstructive azoospermia. However, in men with non-obstructive azoospermia, characterized by severely diminished or absent spermatogenesis, TESA often proves insufficient, necessitating the use of TESE. TESE is a surgical procedure wherein testicular tissue is excised and microscopically examined for sperm retrieval. Despite its efficacy, TESE is more invasive, associated with a longer recovery period, and entails a higher risk of complications compared to TESA. PESA involves the insertion of a fine needle through the scrotal skin to aspirate sperm from the epididymis. However, as a non-visual technique, it limits the ability to directly observe the epididymal tissue, potentially reducing its precision. For scenarios requiring a higher yield or superior quality of sperm, MESA is the method of choice. This microsurgical procedure entails a scrotal incision, enabling direct visualization of the epididymal tissue. Sperm are then carefully identified and aspirated from specific epididymal tubules under microscopic guidance.

After sperm is obtained, different assisted reproductive technologies can be used, such as Intrauterine Insemination (IUI), In Vitro Fertilization (IVF), and Intracytoplasmic Sperm Injection (ICSI)[2]. IUI is the process of injecting concentrated sperm directly into the uterus during ovulation through a thin catheter. This method is simple, less invasive, and suitable for mild infertility, but the success rate is lower than that of IVF and ICSI. IVF is the extraction of eggs from ovarian tissue under vaginal ultrasound guidance, which are then fertilized with sperm in the laboratory (petri dish). The resulting fertilized eggs are cultured in vitro for 3-5 days and then transplanted into the uterus. The difference between ICSI and IVF is that a microneedle is used to inject a single sperm directly into the cytoplasm of a mature egg. ICSI is more suitable for severe male infertility (such as low sperm count, poor motility, or abnormal morphology).

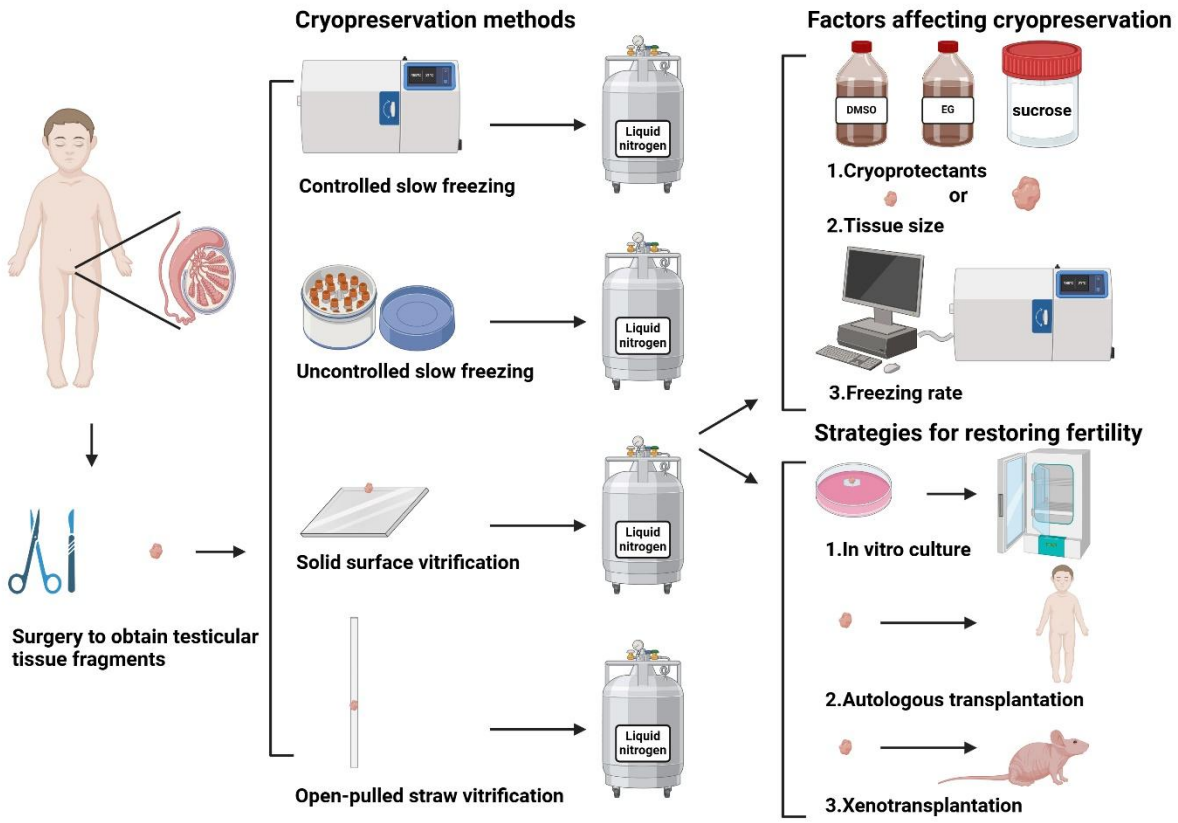
The fundamental premise for the application of assisted reproductive technologies (ART) is the acquisition of mature sperm. However, prepubertal males, whose testicular tissue is still in the developmental stage, cannot produce mature sperm. Consequently, for prepubertal male patients diagnosed with cancer, cryopreservation of testicular tissue before undergoing gonadotoxic radiotherapy or chemotherapy has become a crucial method for preserving future

fertility. This approach offers the potential for restoring reproductive function by preserving spermatogonial stem cells within the testicular tissue. With advancements in radiotherapy and chemotherapy, the survival rate of prepubertal cancer patients has significantly improved. Statistics show that in many European countries, the 5-year survival rate for these patients exceeds 80%. Given the substantial need for fertility preservation within this patient population, there is a pressing demand to establish standardized protocols for the cryopreservation of testicular tissue[3].

Moreover, testicular tissue cryopreservation offers a crucial option for men at risk of premature infertility. For example, male patients with certain genetic disorders, such as idiopathic thalassemia or sickle cell disease, may preserve their fertility by cryopreserving testicular tissue prior to undergoing bone marrow transplantation. Similarly, men with autoimmune diseases, including systemic lupus erythematosus and systemic sclerosis, may consider this technique before initiating therapies that pose a risk to their reproductive potential. This approach provides a proactive means of safeguarding fertility in the face of medical treatments that may adversely affect spermatogenesis.

In patients with Klinefelter syndrome, advancing age leads to fibrosis of the testicular tissue, degeneration of the seminiferous tubules, and loss of germ cells. Post-puberty, these individuals may experience hypogonadism and, in some cases, azoospermia[4,5]. Therefore, cryopreservation of immature testicular tissue in these patients before puberty provides a new possibility for restoring their fertility in the future[6]. However, some studies suggest that testicular tissue cryopreservation may not be ideal for patients with Klinefelter syndrome, as it may not yield better outcomes than sperm extraction from individuals with this condition once their testicular tissue has matured. Evidence indicates that approximately 8% of patients with Klinefelter syndrome retain sufficient spermatogenesis to allow for sperm retrieval[7,8].

Figure 1 Testicular tissue cryopreservation methods and their affecting factors, and strategies for restoring fertility



Cryopreservation method

The cryopreservation of semen is a well-established and highly standardized procedure, widely utilized in both human and animal-assisted reproduction. The technology is mature, and its success rate is high. In contrast, cryopreservation of testicular tissue remains less standardized due to the complexity of its structure, the diversity of cell types, and the insufficient understanding of the mechanisms of cellular and tissue damage during the freezing process. As a result, there is currently no unified, standardized protocol for testicular tissue cryopreservation. The existing methods are primarily categorized into two approaches: slow freezing and vitrification. However, there remains ongoing debate regarding which technique yields superior outcomes in terms of cryopreservation efficacy.

Slow Freezing

Slow freezing methods are typically classified into controlled and uncontrolled slow freezing. Controlled slow freezing utilizes a programmable rate-controlled freezer, allowing precise regulation of the freezing rate. The key advantage of this method is that it ensures the freezing process remains within an optimal range, thereby minimizing the risk of freezing-induced damage. In contrast, uncontrolled slow freezing involves exposure to liquid nitrogen vapors or the -80°C freezer, where the freezing rate is not regulated. Due to the lack of control over the freezing process, variable exposure times to cryoprotectants, and differing levels of operator

expertise, this method generally results in suboptimal freezing outcomes and is considered an alternative to controlled slow freezing[9]. However, the uncontrolled slow freezing method is cheaper and easier to perform outside the laboratory, which may be a better choice for those who study wildlife outdoors[10].

In controlled slow freezing, researchers can optimize the preservation of various cell types within testicular tissue by carefully modulating the freezing rate to achieve optimal results. Studies on the cryopreservation of immature human testicular tissue have indicated that, upon thawing using a controlled slow freezing protocol, the integrity of the seminiferous tubule epithelium is maintained. Furthermore, the concentration of spermatogonia is preserved, along with the expression of DNA replication and repair markers, such as anti-proliferating cell nuclear antigen (PCNA), in both spermatogonia and Sertoli cells[11]. Christine's research demonstrated that even six months after xenotransplantation, controlled slow freezing preserves the proliferative capacity of spermatogonia. However, the differentiation of spermatogonia was suboptimal, with the majority remaining in the pre-leptotene spermatocyte stage, and sperm cells were not observed[12].

In the uncontrolled slow freezing protocol, cryovials are typically placed in an isopropanol container and then positioned in the -80°C freezer. This method results in a freezing rate of approximately -1°C/min until the sample reaches -80°C. Once this temperature is achieved, the cryovials are transferred to a liquid nitrogen tank for long-term storage. Studies have shown that uncontrolled slow freezing, when using the same cryoprotectant solution of 1.5 M DMSO and 0.15 M sucrose, serves as a viable alternative to the controlled slow freezing protocol. After cryopreservation, the ultrastructure of the testicular tissue is preserved with favorable outcomes[9].

Vitrification

Vitrification is the solidification of water or a water-based solution into a glass-like state without the formation of ice crystals[13]. Vitrification is a technique that transforms cells from a liquid phase into a glass-like state by rapidly exposing them to liquid nitrogen. Unlike slow cryopreservation, vitrification necessitates the use of high concentrations of cryoprotectants to increase the viscosity of the cells and prevent ice crystal formation during the freezing process. However, due to the cytotoxicity of cryoprotectants at elevated concentrations, vitrification typically employs a two-step cryoprotectant treatment to mitigate their detrimental effects on cells. The first step involves exposing cells or tissues to a low concentration of cryoprotectant for a brief period, followed by exposure to a higher concentration. Because vitrification involves fewer steps, shorter exposure times, and requires less specialized equipment than slow

freezing, it is considered a faster and simpler method. Initially gaining prominence in the field of female fertility preservation, vitrification has been particularly applied to ovarian tissue[14]. In recent years, researchers have increasingly explored the application of vitrification in the cryopreservation of testicular tissue, yielding promising results. Studies have demonstrated that spermatogonia retains robust proliferative activity following vitrification of testicular tissue[15]. Moacir et al. found that vitrification preserved the morphological integrity of immature testicular tissue, maintaining it similarly to that of fresh tissue[16].

Vitrification techniques primarily include solid surface vitrification (SSV) and open-pulled straw vitrification (OPSV). SSV is a relatively straightforward vitrification method, in which the sample, pre-treated with cryoprotectant, is placed on the surface of a sterile metal block immersed in liquid nitrogen, allowing it to be partially submerged for vitrification[17]. After vitrification, the processed samples are typically placed in pre-cooled cryovials and stored in liquid nitrogen tanks for long-term preservation. OPSV involves placing the cryoprotectant-treated sample on a piece of gauze to remove any excess vitrification medium, after which it is transferred into an open cryotube and immersed in liquid nitrogen. The straw is then inserted into the pre-cooled cryotube, sealed, and stored in a liquid nitrogen tank for long-term storage[18]. Open-tube vitrification has been shown to effectively preserve the integrity of immature mouse testicular tissue[18]. While the open vitrification cryoprotection system offers several advantages, concerns have been raised regarding its safety. Specifically, the use of liquid nitrogen may facilitate the transfer of pathogens, potentially leading to cross-contamination. As a result, the solid surface vitrification system may present a safer alternative for clinical applications[9]. In addition, the study has shown that compared with open vitrification, solid surface vitrification of testicular tissue has higher cell viability and lower cytoplasmic degeneration and vacuolization[19].

Comparison of Slow Freezing and Vitrification

Both slow freezing and vitrification are feasible cryopreservation methods for testicular tissue. For immature mice, uncontrolled slow freezing and solid surface vitrification achieved similar cryoprotective effects and both maintained the integrity of cells and seminiferous tubules of immature testicular tissue[18]. When these two cryopreservation methods were used, there was no significant difference in the mRNA levels of testicular tissue, and the DNA methylation levels were similar[20]. For red-rumped agouti, vitrification increased the number of nucleolar organizer regions (NOR) in spermatogonia and Sertoli cells more than slow freezing, and the increase in the number of NORs can reflect cell proliferation ability[21]. From the analysis of testicular tissue morphology, testicular cell viability, and DNA integrity, both slow freezing and vitrification techniques can effectively protect wild boar testicular tissue[22,23]. Since the low-temperature

resistance of dog primary spermatocytes is worse than that of wild boars, C M Picazo et al. believed that vitrification could better maintain the viability of dog testicular cells and reduce the degree of DNA fragmentation[23]. Interestingly, a study on cryopreservation of gray wolves, also canid, found that slow freezing was superior to vitrification [24]. According to the Worldwide Testicular Tissue Cryopreservation Program, 15 reproductive centers still use slow freezing as the primary method for cryopreservation of immature human testicular tissue[25]. However, due to the relative lack of research on the vitrification of immature human testicular tissue, it is difficult to determine the best solution. Therefore, there is still much room for development in this field of research.

Factors affecting cryopreservation

The optimal cryopreservation protocol for testicular tissue varies across species and remains undefined. Even closely related species within the same family exhibit differing responses to various cryopreservation techniques[26]. Similarly, variations in the parameters of the same cryopreservation technique—such as the concentration and ratio of cryoprotectants, freezing rate, and tissue size—can lead to differing outcomes[27]. Therefore, a large number of experimental samples are needed to optimize the best cryopreservation scheme for different species.

Cryoprotectants

Cryoprotectants are divided into permeable cryoprotectants and non-permeable cryoprotectants. permeable cryoprotectants are small molecules that can penetrate the plasma membrane, form hydrogen bonds with water molecules, lower the freezing point, and thus reduce the formation of ice crystals inside and outside the cell[28]. Non-permeable cryoprotectants include dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol, and 1,2-propanediol (PrOH) [29]. Since a permeable cryoprotectant has increased cytotoxicity at high concentrations, two or more cryoprotectants are often used in cryoprotection to reduce cytotoxicity [22,30].

DMSO is the most common cryoprotectant. The use of DMSO as a cryoprotectant effectively maintained the structural integrity of the spermatogonial germ cells, Sertoli cells, and stromal compartments throughout the processes of freezing, thawing, and subsequent short-term culture in the cryopreservation protocol[31]. After slow freezing with 5% DMSO, cryopreserved human immature testicular tissue was comparable to fresh immature testicular tissue in vitro, with no significant differences in any functional parameters[32]. When 0.7 M DMSO was added for slow freezing, the interstitial cell and tubular epithelial structure of mature human testicular tissue were maintained, but spermatogonia apoptosis increased. When the concentration was

increased to 1.5 M DMSO, spermatogonia apoptosis was reduced while maintaining the original structure[9]. This may be because the increased concentration allowed cells to be more fully dehydrated, thereby reducing the formation of intracellular ice crystals.

EG is another common cryoprotectant. Its cryoprotective effect is not as good as that of DMSO when used alone. Kirsi Jahnukainen et al. compared the cryoprotective effects of 1.4 M EG and 1.4 M DMSO. After xenotransplantation, the graft survival rate of 1.4 M DMSO was 30%, which was higher than that of 1.4 M EG[33]. EG is often used together with DMSO. Compared with vitrification using 3 M DMSO and 3 M EG alone, vitrification using 1.5 M DMSO plus 1.5 M EG can reduce basal cell membrane rupture and contraction, maintain the ultrastructure of seminiferous tubules, increase the proliferation capacity of spermatogonia and Sertoli cells, and improve the mitochondrial activity of testicular tissue[21,34]. In the presence of 20% DMSO, 40% EG can improve the survival rate of immature mouse spermatogonia by more than 20% EG. This may be because the permeability and high osmotic pressure of EG minimize the impact on testicular tissue[35].

Non-permeable cryoprotectants are macromolecules that cannot penetrate the plasma membrane. During the cryoprotection process, they remain in the extracellular matrix and absorb water, thereby indirectly protecting the cells. Non-permeable cryoprotectants include sugars (such as sucrose and trehalose) and high molecular weight polymers (such as polysucrose and polyvinylpyrrolidone) [36]. Compared with permeable cryoprotectants, non-permeable cryoprotectants are less toxic. The addition of non-permeable cryoprotectants reduces the reliance on permeable cryoprotectants while still maintaining good cryopreservation effects. Sugars have multiple functions during cryoprotection. They can increase the viscosity of the cryopreservation solution, thereby reducing the formation of extracellular ice crystals [37]. In addition, sugars can form hydrogen bonds during cryopreservation, thereby protecting the plasma membrane and thus protecting the cells. For example, trehalose can bind to phospholipids in the plasma membrane and protect cells from damage by extracellular ice crystals by forming a glassy shell around the cell [38]. However, non-permeable cryoprotectants have the disadvantage of insufficient permeability, and the testicular tissue structure is complex, with different types of cells closely connected, making it more difficult to diffuse evenly into the tissue.

Sucrose is a non-permeable cryoprotectant that mainly acts on the extracellular matrix. It is rarely used alone and is often used in combination with permeable cryoprotectants such as DMSO or EG. 0.05 M -0.1 M sucrose is often added during slow freezing [39]. Since vitrification requires a viscous and high-concentration cryoprotectant, 0.5 M sucrose is often added[40]. A

recent study showed that adding 0.1 M sucrose during vitrification can obtain a relatively complete basement membrane and a high survival rate of testicular cells[35]. Trehalose is a natural disaccharide that can reduce the fluidity of water and form dihydrate crystals during cryopreservation, thereby improving the tolerance of animals to cryoprotectants[41]. Adding 15% trehalose to 10% DMSO can reduce oxidative stress during cryopreservation of immature testicular tissue in calves and improve the vitality of germ cells[42]. However, some studies have shown that adding 20% trehalose to 10% DMSO can achieve the best cryoprotective effect[43].

Oxidative stress during cryopreservation often leads to the generation of ROS, which can interact with polyunsaturated fatty acids in the plasma membrane, resulting in irreversible damage to its structural integrity[44]. Therefore, incorporating membrane lipids and antioxidants into cryoprotectants may enhance the efficacy of cryopreservation by replenishing membrane components and mitigating oxidative stress. Research by Reyon Dcunha demonstrated that adding soy lecithin, phosphatidylserine, phosphatidylethanolamine, cholesterol, vitamin C, and sodium selenite to cryoprotectants significantly increased the survival rate of testicular cells. Moreover, this approach reduced DNA damage and apoptosis induced by oxidative stress[45].

Melatonin has antioxidant and anti-apoptotic properties, so in recent years, some researchers have explored the possibility of adding melatonin during the cryopreservation process. The receptor-mediated activity of melatonin in testicular tissue involves several pathways. These include binding to melatonin receptors type 1 (MT1), type 2 (MT2), and type 3 (MT3) located on the cell membrane surface, as well as indirect interactions with members of the retinoic acid receptor-related orphan receptor (ROR)/retinoid Z receptor (RZR) family[46]. Melatonin can also exert its effects on testicular tissue through non-receptor-mediated pathways. Excessive production of reactive oxygen species (ROS) disrupts various cellular processes, and melatonin mitigates these effects by indirectly reducing ROS production. This is achieved through the induction of hydrogen peroxide formation, enhancement of antioxidant enzyme activity, and accumulation of intracellular glutathione pools[47,48]. Therefore, incorporating an appropriate amount of melatonin into the cryoprotectant formulation can mitigate the adverse effects of the cryopreservation process on testicular tissue[32,41].

Recent studies have demonstrated that encapsulating cells in hydrogels can facilitate cell vitrification under low-concentration cryoprotectant (CPA) conditions by inhibiting devitrification. This advancement holds significant potential in the field of cell cryopreservation[49]. The hydrogel has a three-dimensional network structure that can limit the formation of ice crystals and simulate the microenvironment of testicular tissue growth, promoting the growth and differentiation of germ cells[50]. Hydrogels, typically composed of natural or synthetic polymer

materials, exhibit excellent biocompatibility and are effective in preventing immune responses[51]. During cryopreservation, alginate hydrogel or gelatin-methacryloyl (GelMA) hydrogel is used. Alginate hydrogel is a natural polysaccharide with high biocompatibility and controllable biodegradability[52]. Incorporating alginate hydrogel can reduce the required concentration of 2.5% DMSO cryoprotectant, thereby minimizing cytotoxicity while maintaining an effective cryoprotective effect[53]. Compared with the traditional cryopreservation with 30% DMSO, the cell apoptosis rate was similar when encapsulated with 20% DMSO combined with 5% gelatin-methacryloyl (GelMA) hydrogel. Still, the testicular tissue morphology was improved, the mitochondrial activity of the testicular tissue was significantly increased, and the antioxidant capacity was enhanced[54]. Hydrogels are also widely utilized in in vitro culture and tissue transplantation, as the incorporation of vascular endothelial growth factor (VEGF) into hydrogels can promote angiogenesis in the embedded tissues[55]. It has been reported that VEGF-loaded nanoparticles incorporated into hydrogels can enhance the recovery of spermatogonia following testicular tissue transplantation[56].

Table 1 Different species are cryopreserved using different cryopreservation methods, cryoprotectants, and tissue sizes.

Species	**method**	**Cryoprotectants**	**size**	**author**
human	Slow freezing	1.5 m DMSO, 0.05 m sucrose and 10% patient serum	1 mg	Aurélie Rives-Feraille[11]
human	Slow freezing	5% DMSO , 5% human serum albumin	2–5 mm^3	Victoria Keros[31]
human	Slow freezing	0.7 mol/L DMSO , 0.1 mol/L sucrose and 10 mg/ml human serum albumin	2–9 mm^3	Jun-Tao Li[57]
human	Slow freezing	5% DMSO and 5% albumin	1–2 mm^3	J M Portela[32]
human	Slow freezing	10% human serum albumin, 1.5 mol/L DMSO, and 0.15 mol/L sucrose	5–6 mm^3	Jose V Medrano[58]
human	Slow freezing	0.7 M DMSO without sucrose and 5 mg/ml human serum albumin or with 0.1 M sucrose.	6–8-mm^3	Y Baert [9]

		1.5 M DMSO, 0.15 M sucrose and 10% HSA		
human	Vitrification	2.1 M DMSO and 2.7 M EG and 20% HSA.	3–4 mm^3	Y Baert [9]
human	Vitrification	15% EG, 15% DMSO , 0.5 M sucrose and 20% HSA	1 × 1 × 3 mm	Jonathan Poels[15]
mouse	Slow freezing	1.5 M DMSO, 0.05 M sucrose, 3.4 mM of (±)-α-Tocopherol, and 10% foetal calf serum	2 or 3 mg	Ludovic Dumont[59]
mouse	Slow freezing	1.5 mol/l DMSO, 0.05 mol/l sucrose and 10% fetal calf serum	Not mentioned	Antoine Oblette[20]
mouse	Vitrification	2.1 mol/l DMSO, 2.7 mol/l ethylene glycol, 0.5 mol/l sucrose and 20% FCS.	Not mentioned	Antoine Oblette[20]
mouse	Vitrification	0.5 M sucrose ,20% FCS	Not mentioned	L Dumont[17]
rats	Vitrification	2.1mol/dm^3DMSO,2.7 mol/dm^3 ethylene glycol, 0.5 mol/dm^3 sucrose and 20% fetal bovine serum	3 × 2 × 1 mm	Gülnaz Kervancıoğlu[19]
rats	Slow freezing	0.7 M DSMO, 0.1 M sucrose and 10 mg/mL human serum albumin	1 mm^3、8 mm^3、27 mm^3	Hong-Xia Wang[60]
rats	Slow freezing	10% fetal calf serum , 0.05M sucrose , DMSO(1.5 M or 3 M), 1,2-propanediol(1.5 M or 3 M)	Not mentioned	A Travers[39]
Red-Rumped Agoutis	Slow freezing	1.5 M DMSO, 1.5 M EG, or 0.75 M DMSO + 0.75 M EG , 10% fetal bovine serum	3 × 1 × 1 mm	Andréia M Silva[21]

Red-Rumped Agoutis	Vitrification	3 M DMSO, 3 M EG, or 1.5 M DMSO + 1.5 M EG，10% fetal bovine serum	3 × 1 × 1 mm	Andréia M Silva[21]
cat	Vitrification	40% EG or 20% EG + 20% DMSO combined with sucrose (0.1 or 0.5 M) or trehalose (0.1 or 0.5 M)	9 mm^3	Carvalho[35]
cat	Vitrification	3% glycerol in the Tris-egg yolk medium	2 × 2 × 2 mm	Beatrice Macente[61]
cat	Vitrification	2.8 M DMSO、2.8 M glycerol、0.5M sucrose、10 % FBS	2-3 mm^3	Olga Amelkina[30]
cat	Vitrification	3% of glycerol or 3% propanediol	0.3cm^3，0.5 cm^3	B l Macente[62]
cat	Vitrification	40% DMSO/GLY, 44% EG/GLY and 44% DMSO/EG.	1 mm^3	Dbc Lima[63]
Macaca fascicularis	Slow freezing	1.4 mol/l DMSO, 10% KSR, 200 mmol/l trehalose, 14 mmol/l hypotaurine, 50 μmol/l necrostatin-1 and 100 μmol/l melatonin.	2–3 mm^3	Sang-Eun Jung[41]
rhesus	Slow freezing	5% fetal bovine serum and 5% DMSO.	9-20 mm^3	Adetunji P Fayomi[64]
Dog and wild boar	Slow freezing	20% foetal bovine serum and 2.8 M DMSO	2 × 2 × 2 mm	CM Picazo[23]
Dog and wild boar	Vitrification	2.1 M DMSO, 2.7 M ethylene glycol, 0.5 M sucrose and 20% foetal bovine serum	1.5×1.5×1.5 mm	CM Picazo[23]

collared peccary	Vitrification	0.25 M sucrose, and 10% fetal bovine serum. isolate cryoprotectants (DMSO or EG) at a 3.0 M concentration or its combination (DMSO/EG) containing a 1.5 M concentration	3 × 3 × 1 mm	Andréia Maria da Silva [34]
collared peccaries	Vitrification	0.25 M sucrose, 10% fetal bovine serum and 3.0 M intracellular cryoprotectant (1.5 M DMSO + 1.5 M Glycerol, 1.5 M DMSO + 1.5 M EG, or 1.5 M EG + 1.5 M Glycerol).	3 × 1 × 1 mm	Andréia Maria da Silva[22]
collared peccaries	Slow freezing	0.25 M sucrose, 10% fetal bovine serum and 1.5 M intracellular cryoprotectant (0.75 M DMSO + 0.75 M Glycerol, 0.75 M DMSO + 0.75 M EG, , or 0.75 M EG + 0.75 M Glycerol)	3 × 1 × 1 mm	Andréia Maria da Silva[22]
bulls	Slow freezing	10% Knockout™ Serum Replacement (KSR)、10% DMSO、5% penicillin-streptomycin with different concentrations of trehalose(5%、10%、15%、20%、25%)	5–8 × 5–7 × 1–2 mm	Wen-Qian Zhu[43]
bovine calf	Slow freezing	(DMSO, EG and glycerol) were prepared at other eight concentrations (2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20%), 10% foetal bovine serum	1–2 mm^3	Hao Li[65]
Goat	Vitrification	40% EG, 18% Ficoll70 and 0.3 M Sucrose	2 × 2 × 1 mm ,3 × 3 × 1 mm, 4 × 4 × 1 mm	Tanushree Patra[66]
grey wolf	Vitrification	20% FBS , 15% EG, 15% DMSO, and 0.5 M sucrose	1-2 mm^3	Christopher S Andrae[24]

grey wolf	Slow freezing	20% FBS and either 15% DMSO or 7.5% DMSO and 7.5% EG	1-2 mm ^{3}	Christopher S Andrae[24]

Freezing Rate

Table 2 summarizes the freezing rates employed by various researchers. These studies differ in their starting temperatures and final cryopreservation tube temperatures, as well as in the freezing rates applied to reach -8°C and -9°C. Some researchers use a freezing rate of -2°C/min, while others apply a slower rate of -0.5°C/min. However, after reaching the inoculation stage, the freezing rate to -40°C typically ranges from -0.5°C/min to -0.3°C/min, with subsequent freezing protocols varying among studies. The optimal freezing rate remains unclear, as the freezing rate curve can be influenced by several factors, including the age of the species, tissue size, and the type and concentration of cryoprotectant. Despite this, one study found that testicular tissue inoculated at -9°C better preserved the structural integrity of the spermatogenic epithelium and the functional integrity of Leydig cells, compared to inoculation at -7°C and -8°C. Furthermore, after 30 days of in vitro culture, testicular tissue cooled at -9°C exhibited seminiferous tubule growth, the germ cell/Sertoli cell ratio, and intratubular cell density more closely resembling those of fresh controls[67].

Table 2 Freezing rates used by different researchers.

author	**Freezing rate**
Ludovic Dumont[59], A Travers[39], Justine Saulnier[68]	start at 5°C, -2°C/min to -9°C, temperature stabilization for 7 min, -0.3°C/min to -40°C, and finally -25°C/min to -150°C.
Hong-Xia Wang[60] ,Jun-Tao Li[57]	start at 0 °C, -0.5 °C /min to -8 °C, and held for 5 min before seeding manually at -8 °C. Then -0.5 °C /min to -40 °C, dehydration at-40 °C for 10 min, and finally -7°C/min to -80 °C.
Aurélie Rives-Feraille[11]	start at 5°C, -2°C /min to -9°C, temperature stabilization for 2 min, -0.3°C /min to -40°C, and finally -10°C/min to -140°C.
Victoria Keros[31], Y Baert[9]	start at 4°C, -1°C/min to 0°C, temperature stabilization for 5 min. Then -0.5 °C /min to -8 °C. At this temperature, the seeding was initiated manually. After holding for 10 min,-0.5 °C /min to -40 °C, dehydration at-40 °C for 10 min, and finally -7°C/min to -70 °C.

Brahim Arkoun[67]	start at 5°C, -2°C /min to -7°C 、-8°C 、-9°C, temperature stabilization for 8 min. -0.3°C/min to -40°C, and finally-25°C/min to -150°C.
Antoine Oblette[20]	start at 5°C , -2°C /min to -9°C, temperature stabilization for 7 min -0.3 °C/min to -40 °C, and finally-10°C/min down to -140°C.

Tissue size

The size of testicular tissue fragments can influence the efficacy of cryoprotection. Due to species-specific differences and the challenges associated with obtaining testicular tissue, the dimensions of the tissue fragments used in cryopreservation can vary across studies. Wang et al. compared the cryoprotective effects of rat testicular tissue of different sizes, specifically 1×1×1 mm (1 mm^3) and 3×3×3 mm (27 mm^3). Their results indicated that tissue fragments measuring 2×2×2 mm (8 mm^3) were most suitable for rat testicular tissue cryopreservation[60]. For goats, the survival rate of testicular tissue with a size of 4 mm^3 after cryopreservation is higher than that of testicular tissue with a size of 9 mm^3 or 16 mm^3[66]. Compared with 0.3 cm^3 fragments, 0.5 cm^3 of domestic cat testicular tissue achieved better results in the cryoprotectant glycerol[69]. In a survey of 16 fertility centers worldwide, it was found that 9 centers cryopreserved testicular tissue fragments measuring ≤5 mm^3, while the remaining centers used tissue fragments ranging in size from 6–20 mm^3[70]. Additionally, during the cryopreservation process, the tissue size should not be excessively large to ensure adequate penetration of the cryoprotectant. However, for subsequent transplantation, the tissue size should not be too small to ensure the viability of the transplant. Therefore, it is essential to determine a critical tissue size specific to each species that optimizes both the cryoprotective effect and the survival of transplanted tissue, ultimately facilitating the production of mature sperm.

Strategies for Restoring Fertility with Immature Testicular Tissue

There are three primary approaches for restoring fertility using cryopreserved immature testicular tissue: in vitro culture of immature testicular tissue to produce mature sperm, autologous transplantation of immature testicular tissue to restore fertility and xenotransplantation of immature testicular tissue for sperm production.

In Vitro Culture

In vitro culture of testicular tissue involves simulating the physiological conditions of the testes in an ex vivo environment. This approach is considered relatively safe, as it eliminates the risk of reintroducing cancer cells into the body. By cultivating the tissue in vitro, mature sperm can be generated, thus avoiding any potential tumor-related risks[71]. At present, human immature

testicular tissue can maintain the integrity of seminiferous tubules and promote the maturation of Sertoli and Leydig cells after long-term in vitro culture[72]. Mature sperm was observed in the testicular tissue of transgender individuals who had undergone long-term hormone therapy, following 34 days of in vitro culture after cryopreservation[73]. Mature sperm have also been successfully observed through in vitro culture of immature testicular tissue from various animal species, including mice[17]. Recent studies have shown that cryopreserved immature testicular tissue of mice can be maintained in organotypic culture for up to 32 days, with spermatogonia differentiating into primary spermatocytes, but complete spermatogenesis was not observed[74].

Common Methods of In Vitro Culture

Testicular tissue culture is typically performed using two main approaches: static culture and microfluidic organ culture. Each method has distinct characteristics, offering optimized in vitro models tailored to different research requirements.

Static Organ Culture

Static organ culture primarily includes gel-supported culture and air-liquid interface culture. Gel-supported culture utilizes three-dimensional (3D) scaffold materials, such as Matrigel, collagen, or gelatin, to provide physical support for the testicular tissue and mimic the chemical signals of the in vivo microenvironment. In this method, the tissue is embedded in the gel, and a culture medium is added on top to cover the tissue, helping to maintain its three-dimensional structure and promote the function of both germ cells and supporting cells. However, long-term culture may lead to nutrient limitations in the central regions of the tissue. In contrast, air-liquid interface culture involves immersing part of the tissue in the culture medium to provide nutrients while exposing the other part to the gas phase, facilitating gas exchange. This approach helps enhance oxygen and nutrient supply to the central regions of the tissue[75]. Takuya Sato et al. demonstrated that static organ culture of testicular tissue can effectively support spermatogenesis[76].

Microfluidic Organ Culture

Microfluidic culture utilizes a miniature fluid channel system to precisely regulate the dynamic flow of culture medium, nutrients, and gases around cells or tissues, mimicking blood flow, material exchange, and signal transmission in the body through dynamic fluid mechanics. When applied to testicular tissue, microfluidic organ culture ensures a more refined supply of nutrients and hormones, thereby supporting spermatogenesis. Compared to static culture, microfluidic culture has been shown to enhance the survival rate of porcine germ cells and improve the efficiency of in vitro germ cell differentiation[77]. A dynamic 3D microbioreactor,

which is a kind of microfluidic organ culture, has been demonstrated to support spermatogenesis in mouse testicular tissue[78].

Common additives for in vitro culture

Previous studies have shown that integrating proteomics technology with the in vitro culture of testicular tissue offers a deeper understanding of cryopreservation processes, as well as the mechanisms underlying germ cell apoptosis and autophagy during culture[79]. Retinol is commonly incorporated into in vitro culture media to enhance spermatogenesis in testicular tissue[80]. Retinol is the active metabolite of vitamin A, directly synthesized by the retinaldehyde dehydrogenases (RALDH)[81]. Retinol can activate the meiosis of germ cells by inducing the expression of retinoic acid gene 8 (Stra8). Therefore, studies have shown that retinol is necessary for the initiation of meiosis[82,83]. Additionally, retinol can influence Sertoli cells, stimulating the synthesis of androgen-binding protein and enhancing the expression of follicle-stimulating hormone (FSH)[84]. Studies have shown that germ cells cultured in organoid systems exhibit a diminished response to retinoic acid stimulation[85], retinoic acid, the biologically active form of vitamin A (retinol), plays a critical role in germ cell differentiation. In rats with vitamin A deficiency, spermatogenesis is disrupted due to impaired spermatogonial differentiation. However, the addition of retinol has been shown to effectively reverse this impairment, restoring spermatogenesis[86].

Adding vitamin E to the testicular tissue culture medium is a promising strategy due to its potent antioxidant properties and cytoprotective effects. Vitamin E enhances the culture environment by reducing oxidative stress, thus improving the survival and functionality of testicular tissue or cells. Specifically, the incorporation of vitamin E into the culture medium has been shown to significantly decrease the accumulation of cytoplasmic ROS during in vitro maturation, promoting improved sperm production[87]. Furthermore, studies have demonstrated that the combined administration of vitamin E and selenium synergistically mitigates testicular dysfunction in mice[88]. As such, the addition of vitamin E to in vitro culture medium may play a crucial role in supporting spermatogenesis.

Autologous testicular tissue transplantation

Autologous testicular tissue transplantation is an emerging experimental technique aimed at restoring fertility by re-transplanting cryopreserved testicular tissue back into the patient's body. This method has garnered significant attention in the field of fertility restoration, offering distinct advantages over other strategies. One of the primary benefits of autologous transplantation is that it preserves the original environment of spermatogonial stem cells. This helps avoid the stress responses typically caused by external environmental changes, thereby reducing the

risk of cell damage. Furthermore, by transplanting testicular tissue in its entirety, spermatogonial stem cells can naturally proliferate and differentiate in the in vivo environment, eliminating the need for complex procedures such as in vitro cell isolation and amplification. In addition, the in vivo environment provides optimal conditions for tissue maturation and functional recovery, further promoting the proliferation and differentiation of germ cells. Notably, autologous transplantation also eliminates the risk of xenogeneic rejection, as the tissue is derived from the patient's body. This approach has already been successfully applied in clinical settings for women, where ovarian tissue transplantation has effectively restored fertility. This evidence supports the potential for similar success in the restoration of male fertility using autologous testicular tissue transplantation[89,90]. However, due to the scarcity of samples and ethical issues, there are few examples of autologous transplantation of adult testicular tissue. A recent case study reported a follow-up study of autologous transplantation of cryopreserved testicular tissue under the scrotal skin in a patient with non-obstructive azoospermia. Six months after the transplant, Doppler ultrasound showed active blood flow in the transplanted tissue. Histological and immunohistochemical analysis showed that the graft survived, with intact and transparent tubules and normal cell organization. Supporting cells and morphologically normal spermatocytes were located near the basement membrane. The graft was still alive, but no sperm was found in the extracted tissue[91]. These results indicate that autologous transplantation technology still needs further research and improvement to better achieve the restoration of fertility function.

The choice of location for testicular tissue transplantation is one of the key factors that determine the success of the technology application and is directly related to the survival rate and functional recovery effect of the transplant. At present, common locations for transplantation include in situ (inside the testicle), subcutaneous scrotum, and other subcutaneous sites. Studies have shown that spermatogenesis can be completed after cryopreserved marmoset testicular tissue is transplanted back to the original site (scrotum); while transplantation to an ectopic site (back skin) leads to spermatogenesis stagnation[92]. A 2019 study further explored the possibility of autologous transplantation of testicular tissue in immature rhesus monkeys, and conducted transplantation experiments under the scrotum and back skin, and successfully obtained sperm and offspring. This study pointed out that the subcutaneous tissue layer of the skin has a higher capillary density. At the same time, local damage caused by suture needles and flap incisions can promote angiogenesis and granulation tissue formation of testicular tissue transplants, thereby enhancing their vascularization ability[64]. Therefore, the selection of the location for autologous testicular tissue transplantation is of great significance in the application of the technology. However, the biological effects of different transplantation locations still need further research to clarify the

best solution and optimize the recovery of reproductive function. Because rhesus monkeys are non-human primates, they are a good reference for studying immature human testicular tissue. The generation of rhesus monkey offspring this time has laid the foundation for future clinical research on humans.

It is important to note that in cases of neoplastic diseases, particularly hematological cancers such as leukemia, there is a potential risk of reintroducing malignant cells into the body through autologous testicular tissue transplantation. Testicular tissue may act as a reservoir for these cancerous cells, raising concerns about the possibility of cancer relapse following transplantation. Studies in mice have demonstrated that as few as 20 leukemia cells, if present in the transplanted tissue, are sufficient to trigger a malignant relapse, highlighting the need for thorough screening and careful consideration when performing autologous transplantation in cancer patients[71]. Currently, histological and immunohistochemical analyses of testicular tissue may not be sophisticated enough to accurately exclude individual malignant cells[93]. Therefore, before autologous transplantation, it is crucial to employ highly sensitive techniques for a comprehensive evaluation of the transplanted testicular tissue. This assessment should include thorough screening for the presence of malignant cells to minimize the risk of reintroducing cancerous tissue into the body. Utilizing advanced diagnostic methods, such as molecular and cellular analysis, can help ensure that the tissue is free from malignancy, thus enhancing the safety and efficacy of the transplantation procedure.

Xenotransplantation

Testicular tissue xenotransplantation refers to the transplantation of testicular tissue from one species into another species. Compared with autologous transplantation, xenotransplantation mainly uses immunodeficient animal models as recipients, which is convenient for observing and analyzing the effects of different conditions and methods on the spermatogenesis process, and provides a basis for the clinical transformation of research results. Currently, there are reports of xenotransplantation of immature testicular tissue from pigs, sheep, and Indian spotted mouse deer (Moschiola indica) into nude mice after cryopreservation, producing mature sperm[94-97].

Due to the immunological disparities between the host and the donor, angiogenesis in xenotransplantation is often less efficient compared to autologous transplantation. However, the success of xenotransplantation largely depends on the graft's ability to rapidly establish vascular connections with the recipient's circulatory system, facilitating the delivery of oxygen, nutrients, and hormones essential for tissue survival and function. In the absence of such vascular integration, the transplanted testicular tissue may experience ischemic stress before

microvascular reconstruction, potentially leading to necrosis of the immature tissue or activation of apoptotic pathways[15]. Therefore, before transplantation, angiogenesis must be considered, and the choice of transplantation site is particularly important. Subcutaneous transplantation is usually better than other sites because the subcutaneous area has dense blood vessels, which is conducive to the rapid growth of new blood vessels. Since the carriers of xenografts are often transplanted into nude mice, which are smaller in size, the back is the main transplantation site. On this basis, vascular endothelial growth factor (VEGF) can promote the proliferation and migration of vascular endothelial cells. Adding it during transplantation may stimulate new angiogenesis and improve the success rate[98].

Hormonal stimulation may also be crucial for sperm development within transplanted testicular tissue. Research has demonstrated that sustained gonadotropin stimulation in the testes of immature monkeys (less than 12 months old) promotes the maturation of Sertoli cells, effectively terminating the unresponsive phase of the germinal epithelium and enabling the progression of full spermatogenesis in the testicular tissue of infant rhesus monkeys[99]. Furthermore, the endocrine environment of the host plays a significant role in the success of transplantation. A recent study involving prepubertal marmoset testicular xenografts demonstrated that the most favorable outcomes for germ cell development were observed in intact male hosts, compared to castrated males or intact females[100]. Therefore, hormone stimulation is also a factor that affects the success of xenotransplantation.

In conclusion, a standardized protocol for the cryopreservation of immature testicular tissue has yet to be established. While many of the studies reviewed focus on animal models, there remains a notable lack of research on the cryopreservation of human immature testicular tissue. Therefore, the development of optimized cryopreservation protocols for human tissue continues to be a critical area of research. Further advancements in this field are essential to achieving more effective cryopreservation methods, which will provide valuable models for studying spermatogenesis and facilitating successful reproductive outcomes.

In this study, to systematically explore the effects of different thawing methods on the physiological functions of tissues and cells during the cryopreservation of immature and mature human testicular tissues, as well as the differences in vitro culture of mature testicular tissues after cryopreservation, we used RNA sequencing technology to conduct an in-depth study of the transcriptomes of these tissues. By analyzing the comprehensive effects of cryopreservation and subsequent in vitro culture on gene expression, we aim to reveal changes in key regulatory genes and their possible biological significance and provide a scientific basis for further optimizing testicular tissue cryopreservation and culture programs.

These findings will also provide new ideas for future technological improvements in the field of fertility preservation and bring better solutions to patients at risk of fertility loss.

3. Comparative Transcriptomic Analyses for the Optimization of Thawing Regimes during Conventional Cryopreservation of Mature and Immature Human Testicular Tissue

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3.1. Abstract

Cryopreservation of human testicular tissue, as a key element of anticancer therapy, includes the following stages: saturation with cryoprotectants, freezing, thawing, and removal of cryoprotectants. According to the point of view existing in “classical” cryobiology, the thawing mode is the most important consideration in the entire process of cryopreservation of any type of cells, including cells of testicular tissue. The existing postulate in cryobiology states that any frozen types of cells must be thawed as quickly as possible. The technologically maximum possible thawing temperature is 100 ◦C, which is used in our technology for the cryopreservation of testicular tissue. However, there are other points of view on the rate of cell thawing, according to how thawing should be carried out at physiological temperatures. In fact, there are morphological and functional differences between immature (from prepubertal patients) and mature testicular tissue. Accordingly, the question of the influence of thawing temperature on both types of tissues is relevant. The purpose of this study is to explore the transcriptomic differences of cryopreserved mature and immature testicular tissue subjected to different thawing methods by RNA sequencing. Collected and frozen testicular tissue samples were divided into four groups: quickly (in boiling water at 100 ◦C) thawed cryopreserved mature testicular tissue (group 1), slowly (by a physiological temperature of 37 ◦C) thawed mature testicular tissue (group 2), quickly thawed immature testicular tissue (group 3), and slowly thawed immature testicular tissue (group 4). Transcriptomic differences were assessed using differentially expressed genes (DEG), the Kyoto Encyclopedia of Genes and Genomes (KEGG), gene ontology (GO), and protein–protein interaction (PPI) analyses. No fundamental differences in the quality of cells of mature and immature testicular tissue after cryopreservation were found. Generally, thawing of mature and immature testicular tissue was more effective at 100 ◦C. The greatest difference in the intensity of gene expression was observed in ribosomes of cells thawed at 100 ◦C in comparison with cells thawed at 37 ◦C. In conclusion, an elevated speed of thawing is beneficial for frozen testicular tissue.

Keywords:

human; testicular tissue; cryopreservation; thawing; RNA sequencing; transcriptomics; differentially expressed genes (DEG); Kyoto Encyclopedia of Genes and Genomes (KEGG); gene ontology (GO); protein–protein interactions (PPI)

3.2. Introduction

Fertility protection of humans and endangered species has always been a hot topic, and spermatozoa cryopreservation has always been a standard method for the preservation of male and animal fertility [1]. However, this technique is not suitable for adolescents and prepubertal individuals who have not yet fully developed gonads. Because they cannot produce mature spermatozoa, testicular tissue cryopreservation is a better fertility preservation technique. For children diagnosed with cancer, the 5-year survival rate has increased to 80%; however, 25% of male survivors still have azoospermia, and testicular tissue cryopreservation is very important for these patients [2–4]. Some adult males require gonadotoxic treatment due to cancer, autoimmune diseases (e.g., systemic lupus erythematosus and systemic sclerosis), and genetic disorders (sickle cell disease thalassemia and idiopathic medulla aplasia). Cryopreservation of testicular tissue for these patients is also an alternative to spermatozoa cryopreservation [5].

Conventional cryopreservation (slow or programmable freezing) is the most commonly used method for testicular tissue preservation [6]. Spermatogenesis is not only related to testicular germ cells but also to testicular somatic cells (e.g., Sertoli cells and Leydig cells) and even the extracellular matrix. The testicular transcriptome represents the sum of all transcripts expressed by different cell groups in testicular tissue. Therefore, RNA sequencing provides the ability to study the entire process of spermatogenesis and the development of testicular cells. Previously published results, including sequencing data, showed that the testis is the organ with the most tissue-specific genes [7,8]. However, there are relatively few RNA sequencing reports on cryopreserved testicular tissue, and only reports on cryopreserved testicular tissue of mice and cats [9,10] are published.

Cryopreservation of human testicular tissue includes the following stages: saturation of cells with permeable cryoprotectants, freezing, thawing, and removal of these cryoprotectants from the cells. As per the perspective prevalent in “classical” cryobiology, the thawing mode holds crucial importance in the cryopreservation technique for all cell types, including testicular tissue cells.

The existing postulate in cryobiology states that any frozen types of cells must be thawed as quickly as possible. The technologically maximal possible thawing rate can be realized with the thawing of cells in boiling water (at 100 ◦C). This mode of thawing was used in our experiments in comparison with the thawing at physiological temperature (37 ◦C).

However, there are other points of view on the rate of cell thawing, according to which thawing should be carried out at physiological temperatures. In fact, there are morphological and functional differences between immature (from prepubertal patients) and mature testicular tissue. Accordingly, the question of the influence of thawing temperature on both types of tissues is relevant.

The purpose of this study is to explore the transcriptomic differences of cryopreserved mature and immature testicular tissue subjected to different thawing methods by RNA sequencing.

3.3. Results

Following the appropriate thawing and removal of cryoprotectants (Figure 1), the cell viability was assessed.

3.3.1. Differentially Expressed Genes (DEG)

At the beginning of the described research, volcano maps were drawn to compare up- and down-regulated differentially expressed genes in tissues of different groups. In comparison with cells of group 2 (slowly thawed mature), 219 differentially expressed genes (DEG) in cells of group 1 (quickly thawed mature) were up-regulated, and 437 DEGs were down-regulated (Figure 2A). At the same time, in comparison with group 2 (slowly thawed mature), the expression of 1073 DEGs in cells of group 4 (slowly thawed immature) was increased, and the expression of 2942 DEGs was decreased (Figure 2B). In comparison with cells of group 1 (quickly thawed mature), 4976 DEGs in group 3 (quickly thawed immature) were up-regulated, and 6071 DEGs were down-regulated. Group 3 has the most intensive DEG expression (Figure 2C). In cells of groups 3 (quickly thawed immature) and 4 (slowly thawed immature), cells which had no significant changes, only one up-regulated and down-regulated DEG was found (Figure 2D).

In contrast with cells of “mature” groups 1 (quickly thawed) and 2 (slowly thawed), cells of “immature” groups 3 (quickly thawed) and 4 (slowly thawed) had 3919 up-regulated DEGs and 6461 down-regulated DEGs (Figure 2E).

3.3.2. Differentially Expressed Genes (DEG) through Enrichment Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways

In comparison with cells of group 2 (slowly thawed mature tissue), cells of group 1 (quickly thawed mature) are mainly enriched in ribosomes, coronavirus disease COVID-19, rheumatoid

arthritis, leishmaniasis, and phagosomes (Figure 3A). Compared to group 2, DEGs in group 4 (slowly thawed immature cells) are mainly enriched in ribosomes, coronavirus disease COVID-19, oocyte meiosis, progesterone-mediated oocyte maturation, and glycolysis/gluconeogenesis (Figure 3B). DEGs in cells of groups 1 and 3 (quickly thawed immature) are mainly enriched in ribosomes, coronavirus disease COVID-19, oocyte meiosis, cellular senescence, and progesterone-mediated oocyte maturation (Figure 3C).

KEGG enrichment analysis of cells from these groups was similar, with ribosomes and COVID-19-related pathways ranked in the top two. DEGs in groups 3 and 4 were mainly enriched in pathways related to oxidative stress, including the NF-κB signaling pathway, the fox O signaling pathway, the p53 signaling pathway, and apoptosis (Figure 3D). Finally, cells from "immature" groups 3 and 4 and from "mature" groups 1 and 2 showed different KEGG pathways, ribosomes, cell cycle, coronavirus disease–COVID-19, glycosaminoglycan degradation, and oocyte meiosis (Figure 3E).

3.3.3. Differentially Expressed Genes (DEG) through Gene Ontology (GO) Enrichment Analysis

Compared to cells from group 2 (slowly thawed mature), cells of group 1 (quickly thawed mature) are mainly enriched in cytoplasmic translation, SRP-dependent cotrans- lational protein targeting to membrane, viral transcription, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, and translational initiation (Figure 4A). The main differences between the GO pathway in cells of group 2 (slowly thawed mature) and group 4 (slowly thawed immature) are spermatogenesis, SRP-dependent co-translational protein targeting to membrane, cytoplasmic translation, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, and viral transcription (Figure 4B). Compared to cells from group 1 (quickly thawed mature), the GO pathway in cells of group 3 (quickly thawed immature) is mainly reflected in spermatogenesis, cytoplasmic translation, SRP- dependent co-translational protein targeting to membrane, cell cycle, and viral transcription (Figure 4C).

GO analysis of cells from these three groups is relatively similar. The GO pathway in cells of group 3 (quickly thawed immature) and group 4 (slowly thawed immature) demonstrated obsolete activation of MAPKKK activity, chromatin silencing, heterochro- matin formation, positive regulation of p38MAPK cascade, and negative regulation of protein kinase activity (Figure 4D).

GO pathways between cells from “immature” groups 3 (quickly thawed immature) + 4 (slowly thawed immature) and cells from “mature” groups 1 (quickly thawed mature) + 2 (slowly thawed mature) include spermatogenesis, cytoplasmic translation, SRP-dependent co-translational protein targeting to membrane, viral transcription, nuclear-transcribed mRNA catabolic process, and nonsense-mediated decay (Figure 4E). The biological process of spermatogenesis is the most enriched, indicating that spermatogenesis in testicular tissue at different developmental stages is more important than the stress caused by different ways of thawing (Figure 3B, C, E). The cellular component and molecular function enrichment of the GO pathway are presented in the Supplementary Materials.

3.3.4. Protein–Protein Interactions (PPI) Network

In cells of groups 1 (quickly thawed mature) and 2 (slowly thawed mature), the ribosome-related protein family has more link nodes; the most important is RPS27A, with a total of 28 link sites, and the remaining RPS14, RPS3A, RPS4X, and RPS6 are also important. PRM2, SPATA3, TNP1, SPATA16, and TNP2 are the more prominent proteins in comparison between cells of groups 2 (slowly thawed mature) and 4 (slowly thawed immature). In the PPI network of cells from groups 1 (quickly thawed mature) and 3 (quickly thawed immature), CSF1R with 22 link sites is the most prominent, and TYROBP, RPS27A, STAT1, and RPS14 are also important (Figure 5).

3.4. Discussion

At present, relevant sequencing studies have been conducted on human cryopreserved spermatozoa, proving that cryopreservation of spermatozoa is a genetically safe fertility preservation method. Moreover, cryopreservation by direct plunging into liquid nitrogen (vitrification) may reduce negative biological changes in spermatozoa in comparison with traditional controlled (conventional) freezing [11]. In our research, RNA sequencing was used to analyze the transcriptional differences produced by different thawing methods in cryopreserved mature and immature testicular tissue in order to optimize the cryopreservation protocol.

3.4.1. Differentially Expressed Genes (DEG)

It was established that DEG expression in cells from group 3 (quickly thawed immature) was less compared to cells from group 4 (slowly thawed immature). The up-regulated genes were H2AC19, and the down-regulated were GADD45B. This indicates that the immature testicular

tissue had less of a response to both different protocols of thawing (slow and quick). H2AC19 is a member of the histone H2A family; its related biochemical processes include the regulatory mechanisms of RNA polymerase I promoter opening and telomere end packaging [12,13]. Recent studies have shown that ubiquitination and acetylation of histone H2A may lead to spermatogenesis disorders in males [14]. The same difference in H2AC19 expression was also observed in cells of groups 2 (slowly thawed mature) and 4 (slowly thawed immature). GADD45B belongs to the growth arrest and DNA damage-induced 45 (GADD45) gene family, whose family members also include GADD45A and GADD45G. These genes are related to physiological and environmental stress and mainly regulate cell proliferation, apoptosis, and DNA damage [15,16]. GADD45B plays a biological role by binding and activating MTK1/MEKK4 kinase and then affecting the p38/JNK pathway [17]. It was reported that stress of epithelial cells due to storage at different temperatures causes changes in GADD45B expression [18].

For quick and slow thawing, the expression of DEGs in cells from groups 1 (quickly thawed mature) and 3 (quickly thawed immature) was much more intensive than that in cells from groups 2 (slowly thawed mature) and 4 (slowly thawed immature), with the number of DEGs being 11,047. It shows that by the quick thawing, the transcriptomic changes of immature cells are more expressed than those of mature cells.

3.4.2. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways

The KEGG pathway enrichment in cells of groups 3 (quickly thawed immature) and 4 (slowly thawed immature) with few DEGs showed that the NF-κB signaling pathway was highly enriched. This pathway is mainly involved in inflammatory and immune responses, and its family members are NF-κB1, NF-κB2, Rel A, Rel B, and c-Rel [19]. During the stress of testicular tissue, NF-κB in Sertoli cells is activated, leading to germ cell apoptosis [20]. Ranked second in KEGG pathway enrichment is the Fox O signaling pathway, which is also mainly involved in apoptosis and oxidative stress. During oxidative stress, such as increased reactive oxygen species, activation of c-Jun N-terminal kinase (JNK) is induced, which leads to translocation of Fox O phosphorylation in the cytoplasm, ultimately affecting downstream targets [21,22]. KEGG analysis showed that cryopreserved immature testicular tissue is intolerant to quick thawing compared to slow thawing.

Except for cells from groups 3 (quickly thawed immature) and 4 (slowly thawed immature), which have fewer DEGs, KEGG pathway enrichment analysis of cells showed that the changes in the ribosome pathway were the most observed. Ribosomes are the site of protein synthesis

within the cell, translating specific genetic information into proteins via messenger RNA. The ribosome-associated pathway includes ribosomal components as well as small nucleolar RNA (snoRNA), ribosomal proteins, and non-ribosomal proteins. The ribosome pathway can cause reproductive toxicity by regulating gene expression, leading to spermatogenesis disorders [23,24]. In addition to occurring during protein synthesis, ribosome collisions often occur when cells are under stress. When cells are subjected to different stresses, it will also cause ribosomes to collide with each other [25]. Current research shows that when cells are subjected to moderate stress, ribosome collision causes the activation of the GCN2-mediated integrated stress response (ISR) signaling pathway. When cells are subjected to high-intensity stress, high-intensity ribosome collisions will activate the p38/JNK-guided signaling pathway [26,27]. However, it is not yet clear which signaling pathway will be activated by cellular stress caused by the different thawing methods used in our research.

The second pathway with significant changes is coronavirus disease (COVID-19). Because some tissue samples were collected during the COVID-19 epidemic, it is uncertain whether the patient was already infected when the sample was collected or whether there was a possibility of contamination during cryopreservation. COVID-19 causes lung damage in patients by binding to the high-affinity angiotensin-converting enzyme 2 (ACE-2) receptors [28]. However, ACE-2 is also abundantly expressed in testicular tissue, especially Leydig cells and spermatogonia. The most abundant protein in Leydig cells is insulin-like factor 3 (INSL3), and its expression was also found to be most significantly decreased in the testicular tissue of infected patients [29]. Pathological examination of testicular tissue of patients who died of serious illness found that the seminiferous epithelium became thinner and the number of apoptotic cells in the seminiferous tubules increased, indicating that infection with COVID-19 can cause spermatogenesis disorders in patients [30].

3.4.3. Gene Ontology (GO)

Overall, the GO analysis showed that the highest enrichment of spermatogenesis was due to different developmental stages of testicular tissue. The remaining most abundant factor is cytoplasmic translation, which is a reaction in which ribosomes mediate protein formation in the cytoplasm. To ensure the stability of the protein state, control mechanisms of the cell's co-translational quality are completed by regulating related mRNA, recycling ribosomes, and degrading nascent polypeptide chains [31]. SRP-dependent co-translational protein targeting the membrane is also a highly enriched biological process. The newly synthesized polypeptide chain usually carries an N-terminal hydrophobic signal sequence. When the polypeptide chain appears in the ribosome polypeptide exit channel, it will be recognized and bound by SRP and

then transported to the endoplasmic reticulum. This pathway can minimize nascent primary proteins misfold and aggregate before reaching the endoplasmic reticulum [32,33].
In relation to our research, different thawing methods will mainly affect biological processes by affecting various stages of protein translation.

3.4.4. Protein–Protein Interactions (PPI)

PPI mapping by DEG cells of groups 1 (quickly thawed mature) and 2 (slowly thawed mature) showed that RPS27A was the most important protein. RPS27A is mainly located in the middle and tail of the spermatozoon and regulates spermatozoon motility. The expression of RPS27A is downregulated in patients with asthenozoospermia and patients exposed to oxidative stress (reactive oxygen species), indicating that it may be a protein marker for detecting spermatozoon motility [34,35]. The difference between cells of groups 1 (quickly thawed mature) and 2 (slowly thawed mature) is mainly due to the application of two different thawing methods. This indicates that RPS27A, which also has great changes caused by temperature stress. The name of PRM2 is Protamine-2, and Protamine-1 can often replace it.

Therefore, maintaining a stable ratio of PRM2 and PRM1 is important to protect the stability of spermatozoon DNA and to reduce the impact of oxidative stress [36,37]. In our study, it was shown that PRM2 plays an important role in the cryopreservation of testicular tissue. CSF1R, which has 22 linking sites, is more active in testicular tissue of different ages by quick thawing. These data are similar to data from previous research regarding increasing CSF1R in mouse testicular tissue with age [38].

A comprehensive analysis using KEGG, GO, and PPI showed that oxidative stress-related pathways and ribosome-mediated protein translation-related pathways play an important role in testicular tissue at different stages and with the use of different thawing methods. Due to the limitation of testicular tissue collection, it cannot be ruled out that the specificity of individual testicular tissue may affect the analysis of sequencing results. In fact, the study of testicular tissue taking into account the development of spermatozoa with the culture of cells in vitro for a certain period with the following sequencing is more informative.

3.4.5. Some Practical Aspects of Described Technology for Cryopreservation

The most common cryo-injuries during thawing include cell osmotic shock and recrystallization. Ice crystals initially melt in the extracellular fluid, causing a decrease in osmotic pressure relative to the intracellular environment. This leads to an influx of extracellular water, resulting

in cellular swelling and eventual disintegration. Simultaneously, water molecules entering the cells can also promote the growth of intracellular ice crystals and cell damage, especially if the thawing rate is slow [39].

The survival of cryopreserved cells is contingent on the warming rate during thawing, and cell death associated with intracellular ice formation is primarily attributed to ice recrystallization rather than the initial nucleation of ice [40,41].

Utilizing a rapid thawing method at 100 ◦C in boiling water allows us to apply the principles of the following cryobiological concept. According to this concept, any biological specimen preserved using any existing cryopreservation techniques should be thawed as swiftly as possible.

In our laboratory conditions, this involves immersing the specimen in boiling water. In theory, an alternative warming medium like boiling oil (ranging from 250 to 300 ◦C) could be used, resulting in a thawing rate several times faster than in boiling water. However, the use of boiling oil for thawing is unsuitable in the sterile environment of a reproductive laboratory.

An essential aspect of thawing in boiling water is the agitation of the water. When a cryo-vial containing a frozen specimen with a temperature of −150 to −130 ◦C is exposed to room temperature for 30 s and then placed in unstirred water, a layer of water with a temperature significantly lower than 100 ◦C forms between the cryo-vial wall and the boiling water. This layer acts as an insulator, reducing the thawing speed. By using a magnetic stirrer to agitate the boiling water, the cool layers of water on the cryo-vial surface are continuously replaced by water with a temperature of +100 ◦C. Our calculations estimate that this element of technology increases the speed of thawing by 15–20%. Additionally, exposing the cryo-vial with biomaterial extracted from liquid nitrogen, which is inherently non-sterile, serves as a surface sterilization step for the cryo-vial, which is crucial in medical technology.

Traditional cryopreservation protocols, which aim to protect cells and prevent intra- cellular crystallization during sub-zero cooling, typically involve the use of permeable cryoprotectants. These cryoprotectants often include three high molecular alcohols such as glycerol, ethylene glycol, propylene glycol, and dimethyl sulfoxide (DMSO). These components constitute 10 to 12% of the total solution, typically comprising either DMSO or a combination of DMSO and one of the glycols [42].

In our protocol, we used multi-cryoprotectants, similar to how we protect ovarian tissue cells. Our data suggest that the protective effect of 12% DMSO alone was inferior to that of a 12% solution supplemented with a combination of cryoprotectants (data not published).

3.5. Materials and Methods

3.5.1. Design of Experiments

A total of 12 human testicular tissue samples (Figure 6) were collected and divided into 4 groups: quickly (in boiling water at 100 ◦C) thawed cryopreserved mature testicular tissue (group 1), slowly (by physiological temperature 37 ◦C) thawed mature testicular tissue (group 2), quickly thawed immature testicular tissue (group 3), and slowly thawed immature testicular tissue (group 4). Mature testicular tissues were collected from 6 adults, and immature testicular tissues were collected from 2 children. In each experimental group, 3 samples were used.

3.5.2. Extraction and Cryopreservation of Testicular Tissue (Equilibration with Cryoprotectants, Thawing and Removal of Cryoprotectants)

The study was conducted in accordance with the Declaration of Helsinki and ap- proved by the Institutional Ethics Committee of Cologne University (protocols 01-106, 12–163, 17-427, 20-1229, code BioMSOTE) and the Bulgarian National Medical Institutional Ethics Committee (Project “Development of new cryopreservation methods to restore testicular function in adult and prepubertal patients with oncological diseases”, approval No. 7-021/2022). The informed consent was obtained from patients whose testicular tissue was collected for this study. All chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA) unless otherwise stated.

All patients underwent a testicular biopsy after the diagnosis of azoospermia as well as for fertility preservation before initiating any therapy carrying a high risk of permanent infertility, such as high-dose chemotherapy. Testicular tissue was obtained from 2 boys and 6 adults aged 3 and 5 and from 34 to 41, respectively.

The procedure of extraction of testicular tissue has been previously described in detail [43–51]. Briefly, a midline incision was made in the scrotum and the testis, and the spermatic cord was removed, preferably from the hemiscrotum, with the larger testis. Tunica vaginalis was opened, and Tunica albuginea was visualized. Under an operating microscope, Tunica albuginea was widely opened in an equatorial plane, preserving the subtunical vessels. After

the opening of Tunica albuginea, testicular parenchyma was examined directly at 12-fold magnification under the operating microscope. Small samples (1–18 mg) were excised by pulling out larger, more opaque tubules from surrounding Leydig cell nodules or hyperplasia in the testicular parenchyma.

Cryopreservation of testicular tissue (Figure 1) was performed according to the previously published protocol for human ovarian tissue [52–55]. Collected testicular fragments were equilibrated for 30 min. in a cryopreservation solution containing 6% dimethyl sulfoxide, 6% ethylene glycol, and 0.15 M sucrose. The cryovials were then placed in an Ice Cube 14S freezer (SyLab, Neupurkersdorf, Austria) for conventional freezing. The freezing procedure is as follows: When the cryovial reaches −7 ◦C, start the freezing process and cool at a rate of −0.3 ◦C per minute until it reaches −33 ◦C. The process usually takes about 90 min. Finally, the cryo-vials were placed in liquid nitrogen for long-term storage. Tissue was thawed with different regimes.

Quick thawing: Thawing of tissue was achieved by holding the cryo-vial for 30 s at room temperature, followed by immersion in a 100 ◦C (boiling) water bath for 60 s, and expelling the contents of the vial into the solution for the removal of cryoprotectants. The exposure time in the boiling water was visually controlled by the presence of ice in the medium; as soon as the ice reached size 2 to 1 mm, the vial was removed from the boiling water, at which point the final temperature of the medium was between 4 and 10 ◦C. Within 5 to 10 s after thawing, the tissue fragments from the cryo-vials were expelled into a 10 mL thawing solution (basal medium containing 0.5 M sucrose) in a 100 mL specimen container (Sarstedt, Nuembrecht, Germany). After the exposure of the tissues to sucrose for 15 min, stepping rehydration of cells was performed, as reported previously [52–55].

Slow thawing: This thawing regime was exactly the same as the quick thawing regime described above, except that the tissues were thawed by immersing the cryovial in a 37 ◦C water bath for three minutes.

3.5.3. Sequencing and Data Extraction

Each sample of testicular tissue was used for RNA extraction with the Trizol method. It was detected that the RIN/RQN of all samples was greater than 4.

Strand-specific transcriptome library construction was completed by enriching mRNA from total RNA, sequenced by DNBSEQ high-throughput platform, and followed by bioinformatics

analysis. The library was validated on the Agilent Technologies 2100 bioanalyzer. The library was amplified with phi29 to make a DNA nanoball (DNB), which had more than 300 copies of one molecule. The DNBs were loaded into the patterned nanoarray, and single-end 50 (pair-end 100/150) base reads were generated using combinatorial Probe- Anchor Synthesis (cPAS). RNA-seq analysis was performed using the Dr. Tom System (https://biosys.bgi.com, accessed on 25 August 2023). The raw data of RNA-seq number is BioProject: PRJNA1030294. It can be downloaded at "Sequence read archive" on the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/1030294, accessed on 14 March 2024). Because the raw data for sequencing contains reads of low quality, adapter contamination, and excessively high levels of unknown base N, these reads need to be removed before data analysis to ensure the reliability of the results.

3.5.4. Differentially Expressed Genes (DEG) Analysis

DEG is the abbreviation of genes, which refers to the detection of genes with different expression levels in different samples. Map clean reads were run to a reference gene sequence (transcriptome), and then gene expression levels for each sample were calculated. Detection of DEG was performed by the DEseq2 method. This DEseq2 method is based on the principle of negative binomial distribution. Our project uses the previously described method [56]. It was analyzed first and maps all candidate genes to each entry in the Gene Ontology database (http://www.geneontology.org/, accessed on 25 August 2023).

R's basis function phyper was used (https://stat.ethz.ch/R-manual/R-devel/library/ stats/html/Hypergeometric.html, accessed on 25 August 2023) to calculate the p-value. Then the p-value is corrected through multiple tests, and the corrected package is the q-value (https://bioconductor.org/packages/release/bioc/html/qvalue.html, accessed on 25 August 2023). Finally, q-value (corrected p-value) ≤ 0.05 was used as the threshold, and the KEGG and GO term that satisfied this condition was defined as the KEGG and GO term that was significantly enriched in candidate genes. PPI analysis of differentially expressed genes was based on the STRING database with known and predicted protein- protein interactions.

3.6. Conclusions

No fundamental differences in the quality of cells of mature and immature testicular tissue after cryopreservation were found. Generally, thawing of mature and immature testicular tissue was more effective at 100 ◦C. The greatest difference in the intensity of gene expression was

observed in ribosomes of cells thawed at 100 ◦C in comparison with cells thawed at 37 ◦C. In conclusion, an elevated speed of thawing is beneficial for frozen testicular tissue.

Funding:

These investigations were supported by the China Scholarship Council for Cheng Pei (No. 202208080057) and by the Bulgarian National Science Foundation (Grant KP-06-N51/11).

Institutional Review Board Statement:

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Cologne University (protocols 01-106, 12–163, 17-427, 20-1229, code BioMSOTE) and Bulgarian National Medical Institutional Ethics Committee (Project "Development of new cryopreservation methods to restore testicular function in adult and prepubertal patients with oncological diseases", approval No. 7-021/2022).

Data Availability Statement:

The raw data of RNA-seq can be downloaded at "Sequence read archive" on National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/1030 294, accessed on 14 March 2024).

Acknowledgments:

The authors would like to thank Elvira Hilger and Mohammad Karbassian for their technical assistance.

Conflicts of Interest:

The authors declare no conflict of interest.

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3.8. APPENDIX

Figure

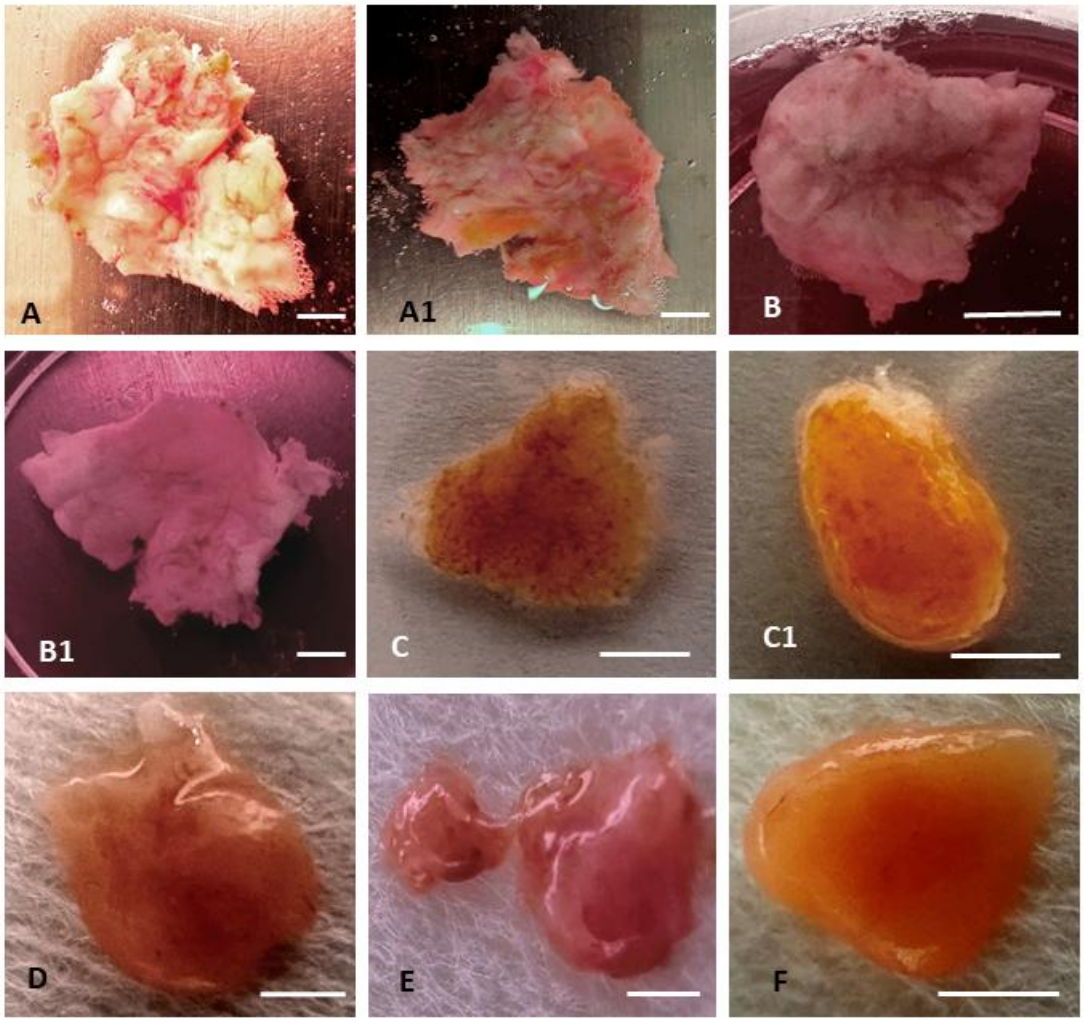

Figure 1. Fragments of mature and immature testicular tissue after cryopreservation. (A) Fragment of mature testicular tissue of patient B. from the tunica albuginea side (“outer” layer) after cryopreservation. (A1) The same fragment of tissue of patient B. from the seminiferous tubules side (“inner” layer). (B) Fragment of mature testicular tissue from patient D. in a hypertonic solution (0.5 M sucrose) during removal of cryoprotectants shows tissue compaction as a result of cell dehydration. (B1) The same fragment of testicular tissue of patient D. in isotonic solution after removal of cryoprotectants. (C) Fragment of immature testicular tissue from patient C. after cryopreservation. (C1) Demonstration photographs: the same dehydrated fragment on filter paper of immature testicular tissue from patient C. after cryopreservation. (D–F) Demonstration photographs: dehydrated fragments on filter paper of mature testicular tissue from patients K., P., and M. after cryopreservation. Scale Bar = 1 mm.

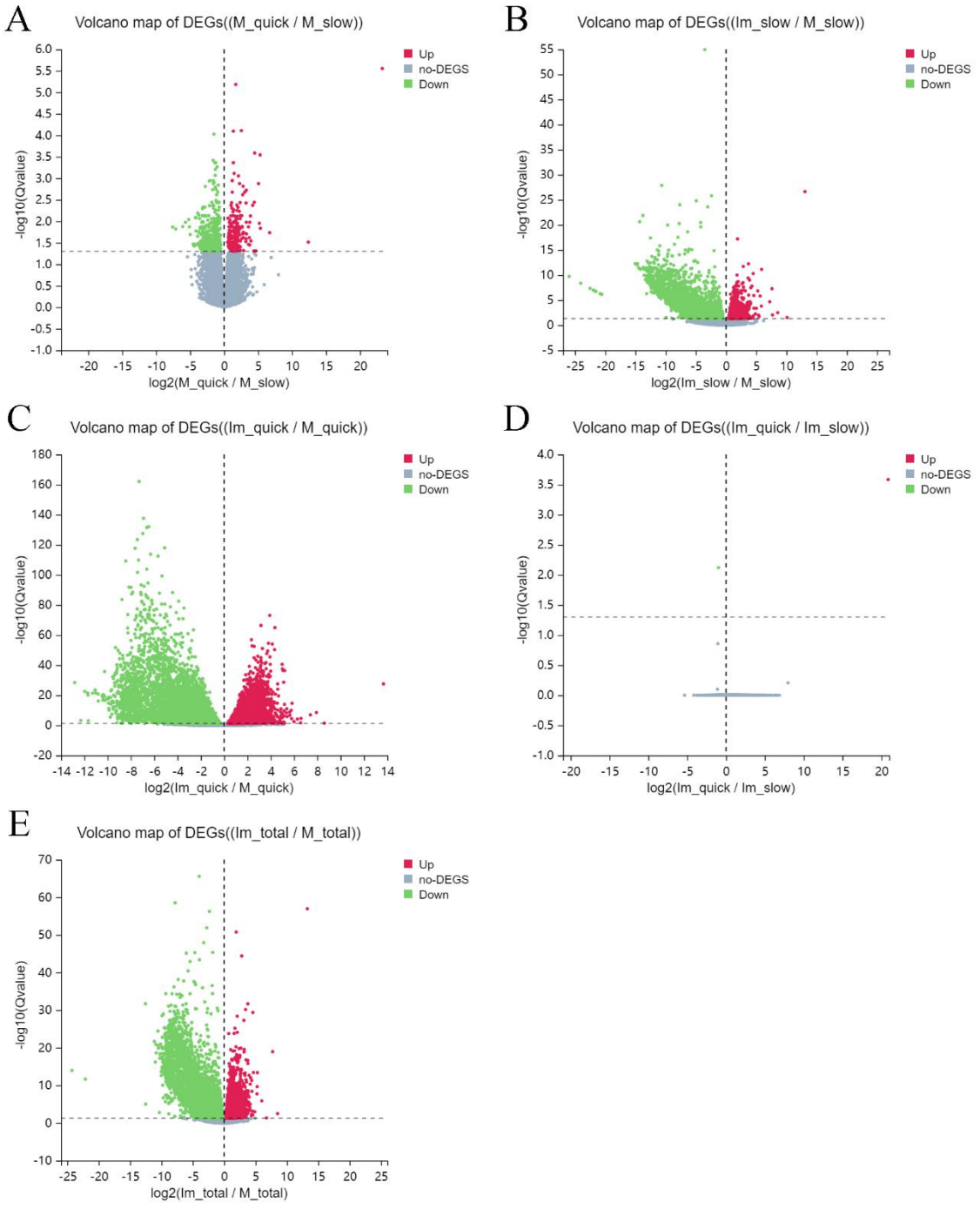

Figure 2. Volcano map of differentially expressed genes (DEG) after different regimes of thawing. (A) DEG volcano map: cells of group 1 (quickly thawed mature) vs. cells of group 2 (slowly thawed mature). (B) DEG volcano map: cells of group 4 (slowly thawed immature) vs. cells of group 2 (slowly thawed mature). (C) DEG volcano map: cells of group 3 (quickly thawed immature) vs. cells of group 1 (quickly thawed mature). (D) DEG volcano map: cells of group 3 (quickly thawed immature) vs. cells of group 4 (slowly thawed immature). (E) DEG volcano map: cells of group 3 (quickly thawed immature) + group 4 (slowly thawed immature) vs. cells of group 1 (quickly thawed mature) + group 2 (slowly thawed mature).

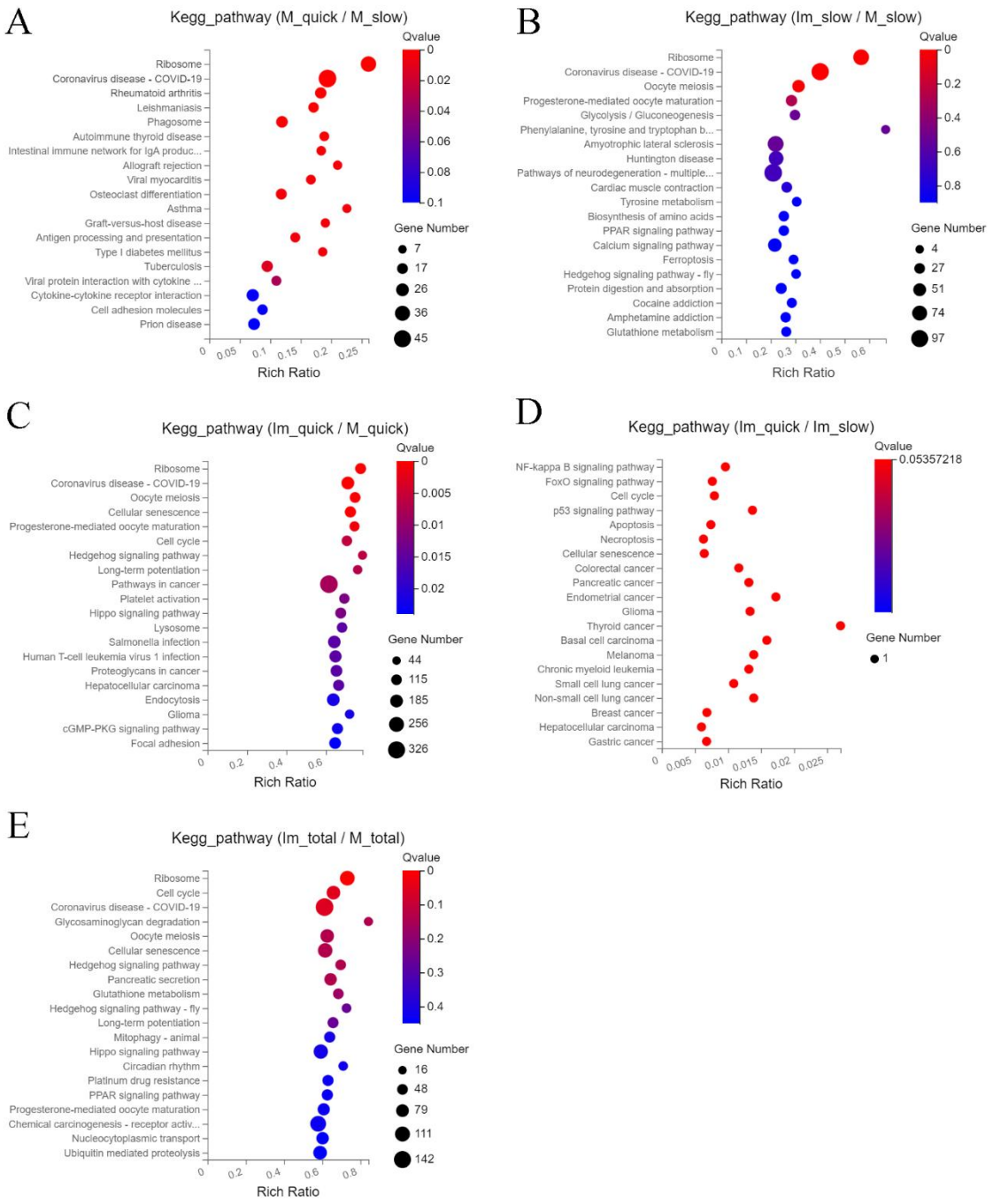

Figure 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment bubble chart of Differentially Expressed Genes (DEG). (A) KEGG pathway chart of DEG in cells of groups 1 (quickly thawed mature) and 2 (slowly thawed mature). (B) KEGG pathway chart of DEGs in cells of groups 2 (slowly thawed mature) and 4 (slowly thawed immature). (C) KEGG pathway chart of DEGs in cells of groups 1 (quickly thawed mature) and 3 (quickly thawed immature). (D) KEGG pathway chart of DEGs in cells of groups 3 (quickly thawed immature) and 4 (slowly thawed immature). (E) KEGG pathway chart of DEGs in cells of groups 3 (quickly thawed immature) + 4 (slowly thawed immature) and groups 1 (quickly thawed mature) + 2 (slowly thawed mature).

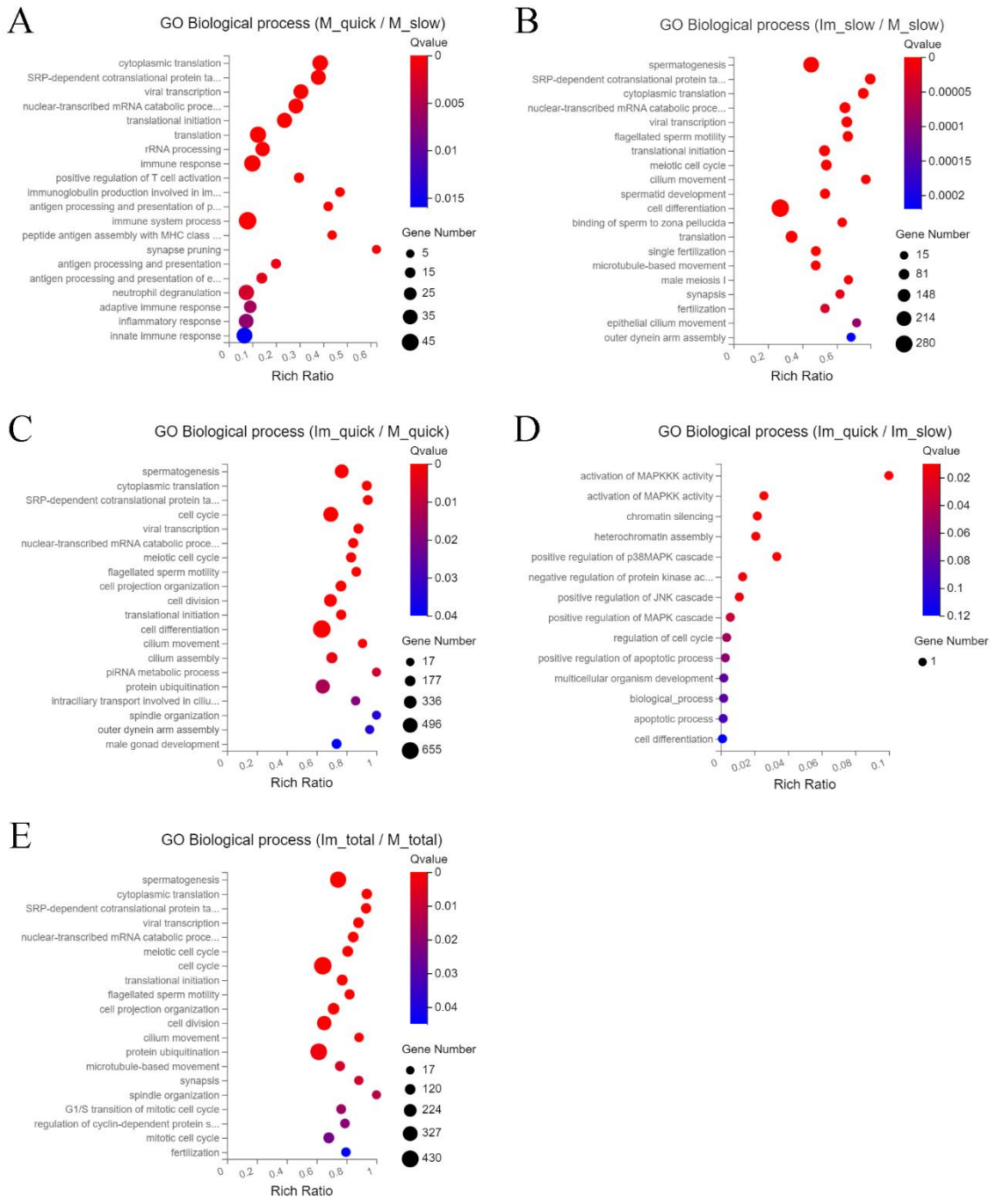

Figure 4. Visualization of Gene Ontology (GO) enrichment bubble chart for cells of different groups. (A) GO enrichment bubble chart for groups 1 (quickly thawed mature) and 2 (slowly thawed mature). (B) GO enrichment bubble chart for groups 2 (slowly thawed mature) and 4 (slowly thawed immature). (C) GO enrichment bubble chart for groups 1 (quickly thawed mature) and 3 (quickly thawed immature). (D) GO enrichment bubble chart for groups 3 (quickly thawed immature) and 4 (slowly thawed immature). (E) GO enrichment bubble chart for groups 3 (quickly thawed immature) + 4 (slowly thawed immature) and groups 1 (quickly thawed mature) + 2 (slowly thawed mature).

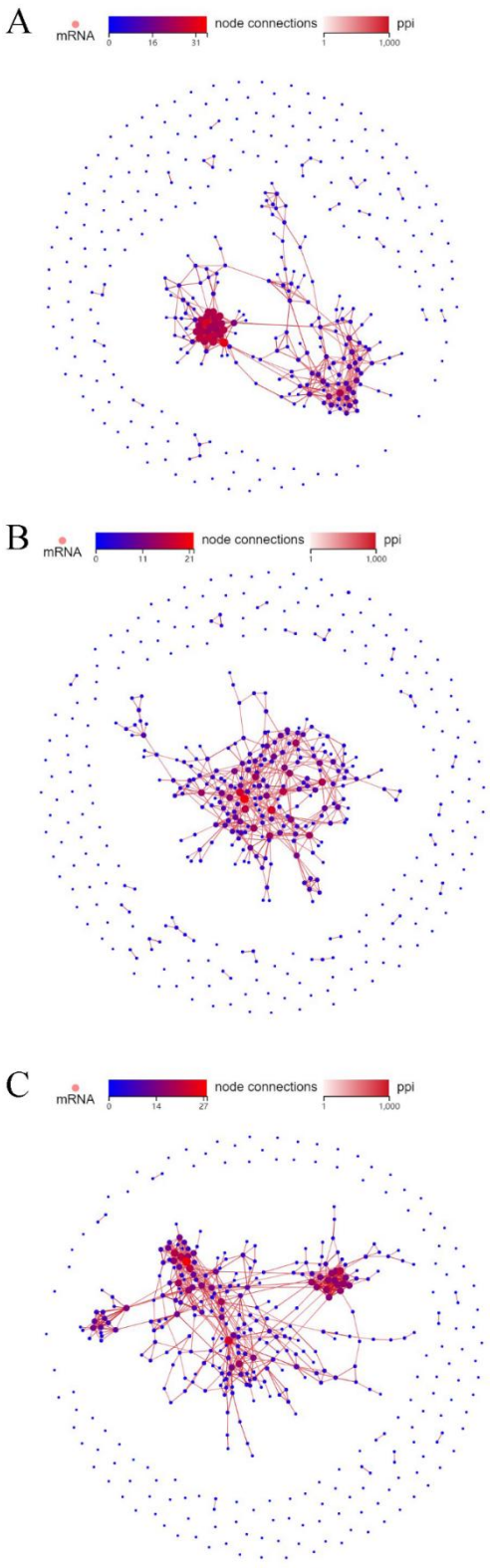

Figure 5. Protein–protein interactions (PPI) networks. (A) PPI network for cells of groups 1 (quickly thawed mature) and 2 (slowly thawed mature). (B) PPI network for cells of groups 2 (slowly thawed mature) and 4 (slowly thawed immature). (C) PPI network for cells of groups 1 (quickly thawed mature) and 3 (quickly thawed immature).

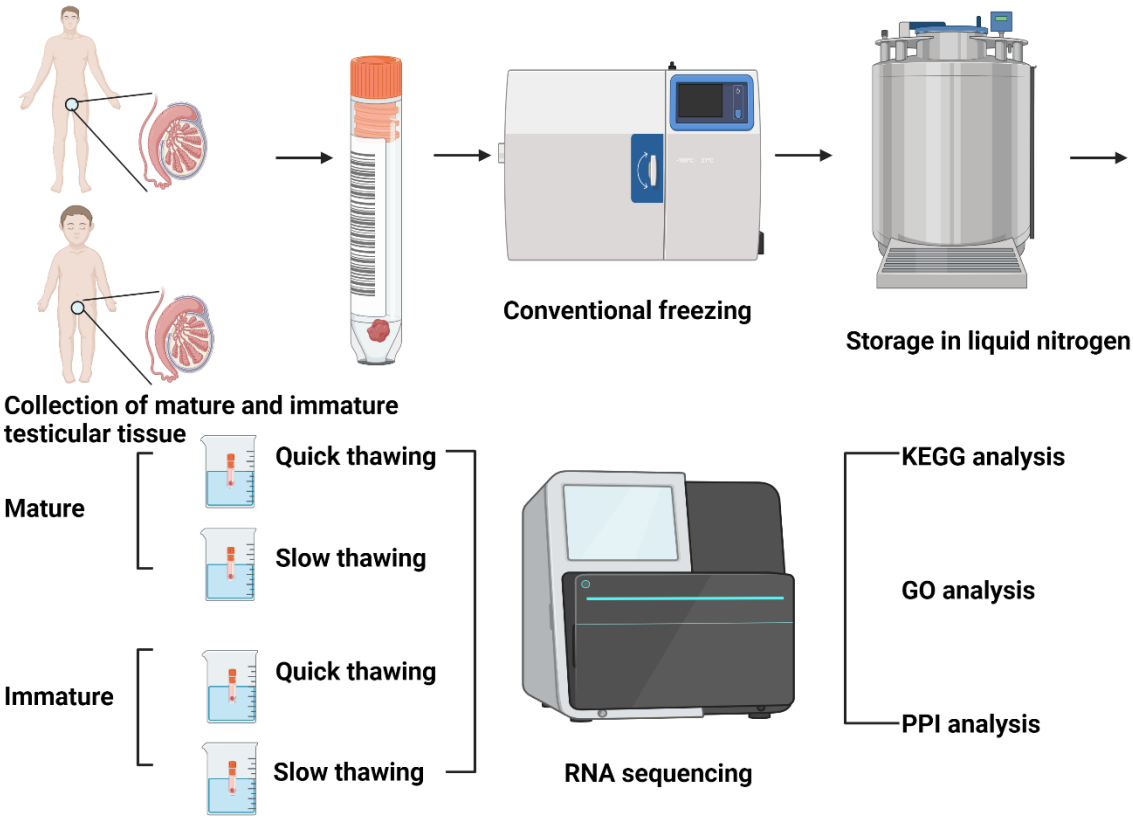

Figure 6. Design of experiments. (KEGG) Kyoto Encyclopedia of Genes and Genomes, (GO) Gene Ontology, (PPI) protein–protein interaction.

4. Transcriptomic Differences by RNA Sequencing for Evaluation of New Method for Long-Time In Vitro Culture of Cryopreserved Testicular Tissue for Oncologic Patients

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4.1. Abstract

Background: Earlier studies have established that culturing human ovarian tissue in a 3D system with a small amount of soluble Matrigel (a basement membrane protein) for 7 days in vitro increased gene fusion and alternative splicing events, cellular functions, and potentially impacted gene expression. However, this method was not suitable for in vitro culture of human testicular tissue. Objective: To test a new method for long-time in vitro culture of testicular fragments, thawed with two different regimes, with evaluation of transcriptomic differences by RNA sequencing. Methods: Testicular tissue samples were collected, cryopreserved (frozen and thawed), and evaluated immediately after thawing and following one week of in vitro culture. Before in vitro culture, tissue fragments were encapsulated in fibrin. Four experimental groups were formed. Group 1: tissue quickly thawed (in boiling water at 100 ◦C) and immediately evaluated. Group 2: tissue quickly thawed (in boiling water at 100 ◦C) and evaluated after one week of in vitro culture. Group 3: tissue slowly thawed (by a physiological temperature 37 ◦C) and immediately evaluated. Group 4: tissue slowly thawed (by a physiological temperature 37 ◦C) and evaluated after one week of in vitro culture. Results: There are the fewest differentially expressed genes in the comparison between Group 2 and Group 4. In this comparison, significantly up-regulated genes included C4B_2, LOC107987373, and GJA4, while significantly down-regulated genes included SULT1A4, FBLN2, and CCN2. Differential genes in cells of Group 2 were mainly enriched in KEGG: regulation of actin cytoskeleton, lysosome, proteoglycans in cancer, TGF-beta signaling pathway, focal adhesion, and endocytosis. These Group 2- genes were mainly enriched in GO: spermatogenesis, cilium movement, collagen fibril organization, cell differentiation, meiotic cell cycle, and flagellated spermatozoa motility. Conclusions: Encapsulation of testicular tissue in fibrin and long-time in vitro culture with constant stirring in a large volume of culture medium can reduce the impact of thawing methods on cryopreserved testicular tissue.

Keywords:

human testicular tissue; cryopreservation; in vitro culture; fibrin granules; RNA sequencing; transcriptomics

4.2. Introduction

The preservation of male fertility often involves cryopreservation of spermatozoa, a process wherein these spermatozoa can be obtained either through ejaculation or following a testicular biopsy. For prepubertal cancer patients lacking active spermatogenesis, traditional semen cryopreservation proves ineffective due to the absence of mature spermatozoa. Alternative methods, such as cryopreservation of spermatogonial stem cell-rich testicular cell or testicular tissue cryopreservation, become paramount in preserving fertility for these individuals.

In addition, while the enzymatic digestion of testicular tissue to isolate spermatogonial stem cells may compromise cell viability, cryopreservation of testicular tissue preserves crucial cell–cell and cell–matrix interactions [1,2]. The latter method emerges as potentially superior. Moreover, testicular tissue cryopreservation holds promise for adult patients grappling with genetic disorders or autoimmune diseases [3].

Testicular tissue in vitro culture stands as a pivotal method for unraveling the complexities of spermatogenesis and addressing male infertility concerns. Given the scarcity of human samples, research predominantly relies on animal models such as mice and pigs for studying in vitro culture and observing spermatogenesis dynamics [4,5]. It was reported that an attempt was made to culture in vitro cryopreserved human immature testicular tissues for 32 days, albeit without observing spermatogenesis [6].

The two primary approaches to in vitro culture of testicular tissue are the microfluidicbased dynamic culture system and the agarose-based static culture system [7]. Microfluidic devices crafted from polydimethylsiloxane (PDMS) hold promise for enhanced spermatogenesis efficiency owing to PDMS's inherent affinity for biological samples [8]. However, the widespread adoption of this method faces challenges due to the demanding operational requirements of the device and the high cost of PDMS chips. In this study, drawing upon our team's prior expertise with artificial ovaries, testicular tissue was encapsulated in TISSEEL Fibrin and subjected to in vitro culture [9].

RNA sequencing has significantly advanced understanding of testicular tissue development and pathology. Investigations performed on murine testicular tissue illuminate the dynamics of gene expression, across various stages of spermatogenesis. The minimal impact of cryopreservation on gene expression was shown [10].

We had the following reason for performing the experiments described in this article: we had earlier tested the protocol of in vitro culture of cryopreserved human ovarian tissue [11]. In short, cryopreservation (freezing and thawing) of tissue was performed by described protocol [12–15].

Experimental group pieces were placed on a floating membrane filter, after which a 3D culture system was created. To achieve this, Corning Matrigel Matrix, a soluble extract of basement membrane protein, was diluted with IVG medium (1:1), and 5 µL of the mixture was placed on the filter to form a small drop (one drop per membrane). Ovarian pieces were then placed on each drop (one piece per drop) and cultured for 7 days [11].

After 7 days of in vitro culture, the morphology of the ovarian tissue changed compared to un-cultured tissue. A dense fibrotic capsule formed, along with a slight reduction in volume [11]. The originally sharp edges of the ovarian cortical pieces became rounded, losing their angular shape. Additionally, cortical slices showed increased fibrosis, more follicular necrosis, and reduced living space for follicles, impacting the development of mature follicles [11]. At the DNA and RNA levels, gene fusion events occurred across nearly every chromosome, involving multiple genes and resulting in new patterns of gene expression and functional changes. These negative alterations [11] provided the basis for the investigations presented here.

Our investigations aimed to test the new method of testicular fragments thawed with two different regimes and long-time in vitro culture, with the evaluation of transcriptomic differences by RNA sequencing.

4.3. Materials and Methods

Unless specified otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

4.3.1. Design of Experiments

Six testicular tissue samples were collected, cryopreserved (frozen and thawed) [12–15] and evaluated. Four groups were formed.
Group1: tissue quickly thawed (in boiling water at 100 °C) and immediately evaluated.
Group 2: tissue quickly thawed (in boiling water at 100 °C) and evaluated after one week of in vitro culture.

Group 3: tissue slowly thawed (by a physiological temperature 37 °C) and immediately evaluated.
Group 4: tissue slowly thawed (by a physiological temperature 37 °C) and evaluated after one week of in vitro culture.
Three samples in each experimental group were used (Figure 1).

4.3.2. Collection of Samples

The experiments were performed in accordance with the Declaration of Helsinki and approved by the Bulgarian National Medical Institutional Ethics Committee (project “development of new cryopreservation methods to restore testicular function in adult and prepubertal patients with oncological diseases”, approval No. 7-021/2022) and the Institutional Ethics Committee of Cologne University (protocols 01-106, 12–163, 17-427, 20-1229, code BioMSOTE).

The technology tested in our studies is being developed for cancer patients. However, the testicular tissue fragments used for the experiments were obtained from patients involved in a fertility treatment program. Five patients were diagnosed with obstructive azoospermia; one patient was diagnosed with restricted azoospermia. Informed consent was obtained from patients whose tissue was collected for this study. Testicular tissue fragments (3 to 12 mm3) were obtained from six patients aged from 32 to 42 (median age 36.5 years). Two tissue fragments from each patient were used for experiments (a total of twelve tissue samples were collected for the research).

The procedure of extraction of testicular tissue has been previously described in detail [16–18]. A midline scrotal incision was performed, and the testis with the spermatic cord was removed, typically from the hemiscrotum containing the larger testis. The tunica vaginalis was opened to reveal the tunica albuginea, which was incised along the equatorial plane under a microscope, ensuring the preservation of subtunical vessels. After exposing the testicular parenchyma at 12× magnification, small tissue samples (3–10 mg) were excised by isolating thicker, opaque tubules from areas of Leydig cell nodules or hyperplasia.

4.3.3. Cryopreservation (Freezing and Thawing)

Testicular fragments were equilibrated for 30 min in cryovials containing a cryopreservation solution with 6% ethylene glycol (EG), 6% dimethyl sulfoxide (DMSO), and 0.15Msucrose. The cryovials were then placed in an Ice Cube 14S freezer (SyLab, Neupurkersdorf, Austria) for conventional freezing, as described earlier for ovarian and testicular tissues [12–15]. The

cryopreservation program consisted of the following stages: (1) initial cooling from −6 ◦C to −8 ◦C; (2) subsequent cooling from −6 ◦C to −34 ◦C at a rate of 0.3 ◦C/min; and (3) plunging the cryovials into liquid nitrogen at −34 ◦C. The protocol also included an auto-seeding step during the initial cooling phase. Subsequently, the cryovials were stored in liquid nitrogen for long-term preservation.

Tissues were thawed with two regimes:

Quick thawing: Cryovials were first exposed to room temperature for 30 s and then immersed in a 100 ◦C water bath for 60 s. The exposure time in boiling water was visually monitored using ice presence as a gauge. When ice reached 2-1mm, the cryovial was removed, resulting in a final medium temperature of 4 to 14 ◦C. Within 5 to 10 s after thawing, tissue fragments from the cryovials were transferred into a 10 mL thawing solution (basal medium with 0.5 M sucrose) in a 100 mL specimen container (Sarstedt, Numbrecht, Germany). The tissues were exposed to the sucrose solution for 15 min, followed by a stepwise rehydration process, as previously described [12–15].

Slow thawing: Cryovials were first exposed to room temperature for 30 s and then immersed in a 37 ◦C water bath for 3 min. The subsequent thawing steps were identical to those used in the quick thawing regime described earlier.

4.3.4. 3D In Vitro Culture

The protocol for in vitro culture was previously used for the preparation of artificial ovaries [9].

TISSEEL Fibrin Sealant (Baxter International Inc., Deerfield, IL, USA) was employed for the encapsulation of testicular tissue for subsequent in vitro culture and analysis. The fibrin sealant comprises two main components: thrombin and fibrinogen, used at final concentrations of 10 IU/mL and 45.5 mg/mL, respectively, to achieve optimal gel formation and encapsulation. The two components were quickly mixed in an Eppendorf tube using a pipette and vortexed. Then 100 µL of solution was dropped onto testicular tissue to form a nearly gel-like mixture (Figure 2). Upon completion of gelation, the fibrin gel formed was gently peeled off using sterile tweezers to avoid any damages in tissue during in vitro culture.

The encapsulated tissue was then immediately transferred into a 700 mL cell culture flask (Greiner Bio-One GmbH, Frickenhausen, Germany) containing 70 mL of culture medium. The 7-day culture of testicular tissue was conducted in alpha-modified Eagle’s minimum essential

medium (α-MEM, Life Technologies, Carlsbad, CA, USA) supplemented with 15% fetal calf serum, 0.1 mg/mL streptomycin, and 100 IU/mL penicillin. The cultures were maintained in a humidified incubator at 34 ◦C with 5% CO2 and agitated at 75 oscillations per minute using a rotating shaker.

4.3.5. Histology

Testicular tissues were fixed in 3.5% paraformaldehyde for 12 h at 4 ◦C and then embedded in paraffin wax. Sections with a thickness of 5 µm were cut, and every 10th section from each sample was mounted on a glass slide and stained with hematoxylin and eosin [19]. The section was subjected to morphological analysis of tissue development and viability under microscope Nikon Diaphot 300 (Nikon, Tokyo, Japan).

4.3.6. Data Extraction and Sequencing

Each testicular tissue sample underwent RNA extraction using the Trizol method. Subsequently, a strand-specific transcriptome library was created by isolating mRNA from total RNA. Sequencing was performed using the DNBSEQ high-throughput platform, followed by extensive bioinformatics analysis. RNA-seq data were analyzed using the Dr. Tom System (https://biosys.bgi.com accessed on 25 August 2023). Download the results of KEGG and GO related enrichment through this system. The raw RNA-seq data are accessible under BioProject: PRJNA1030294 and can be downloaded from the “Sequence read archive” on the National Center for Biotechnology Information (https: //www.ncbi.nlm.nih.gov/bioproject/1030294 accessed on 14 March 2024).

4.4. Results

4.4.1. Morphology

In the cryopreservation process of testicular tissue presented in Figure 2(A2–C2), a halo (crown) was observed around the fragments. The process of removal of cryoprotectants occurred because of the dehydration of cells in 0.5 M sucrose when cryoprotectants were removed from them. The halo (crown) is explained by the different densities of sucrose and cryoprotectant solutions, which leave cells because of an osmotic reaction in cell membranes. It also illustrated the process wherein cryopreserved fragments of testicular tissue were sealed in fibrin granules before long-time in vitro culture (Figure 2(A4–C4)). Cryopreserved fragments

of testicular tissue sealed in fibrin granules after long-time in vitro culture can be seen in Figure 3.

HE-staining of testicular tissue revealed improved morphology in specimens subjected to quick thawing followed by in vitro culture compared to quick thawing and immediate evaluation. In cells of Group 2 (quick thawing and in vitro culture) in comparison with Group 1 (quick thawing), the gap between the cells of the seminiferous tubules and the basement membrane was reduced, the basement membrane rupture was improved, and the nuclear condensation of spermatogonia was reduced (Figure 4(A1,A2,B1,B2)). Similarly, tissues after slow thawing followed by in vitro culture exhibited better morphology than those subjected to cells of group with slow thawing (Figure 4(C1,C2,D1,D2)). After in vitro culture, the contracted basement membrane was partially restored, the spermatogonia and supporting cells were plumper, and there were fewer gaps between adjacent seminiferous tubules (Figure 4).

4.4.2. Differentially Expressed Genes

In cells of Group 2 (quick thawing and in vitro culture), 5513 up-regulated and 6422 down-regulated differential genes were detected in comparison with cells of Group 1 (quick thawing). Meanwhile, cells from Group 4 (slow thawing and in vitro culture) displayed 4533 genes with decreased expression and 3030 genes with increased expression compared to Group 3 cells (slow thawing). Comparison of Group 2 cells (quick thawing and in vitro culture) and Group 4 cells (slow thawing and in vitro culture) revealed 21 differential genes with increased expression and 11 genes with decreased expression in Group 2 (Figure 5A–C). In this comparison, significantly up-regulated genes included C4B_2, LOC107987373, and GJA4, while significantly down-regulated genes included SULT1A4, FBLN2, and CCN2.

Aggregate analysis of tissues thawed by both methods indicated that in cells from Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) 4818 up-regulated and 7660 down-regulated differential genes were detected in contrast with cells of Group 1 (quick thawing) and Group 3 (slow thawing). Also, it was established that up-regulated genes included UTP14C, GSTT1, and SCG2, and down-regulated genes included GAGE12E, GAGE12H, and CXorf51B (Figure 5D).

4.4.3. Enrichment Analysis of KEGG Pathways

Comparative KEGG pathway analysis revealed distinct patterns between cells from Group 2 (quick thawing and in vitro culture) and Group 1 (quick thawing), where the enriched pathways

in Group 2 included lysosome, regulation of actin cytoskeleton, cellular senescence, MicroRNAs in cancer, and proteoglycans in cancer (Figure 6A).

Similarly, in comparison with cells of Group 3 (slow thawing), cells of Group 4 (slow thawing and in vitro culture) exhibited enrichment in pathways such as lysosome, focal adhesion, and calcium signaling pathway (Figure 6B). It was noted that the lysosome pathway showed the highest enrichment in both comparisons. In comparison with cells of Group 4, cells of Group 2 displayed enrichment in pathways like cytokine–cytokine receptor interaction, IL-17 signaling pathway, viral protein interaction with cytokine and cytokine receptor, Aldosterone synthesis and secretion, and protein digestion and absorption (Figure 6C). Comprehensive analysis across all groups revealed that the “thawing and in vitro culture” groups (Group 2 and Group 4) exhibited KEGG pathways, including regulation of actin cytoskeleton, lysosome, focal adhesion, TGF-beta signaling pathway, and proteoglycans in cancer, emphasizing the impact of in vitro culture on these biological processes (Figure 6D).

4.4.4. GO Biological Process Enrichment Analysis

In comparison with cells from Group 1 (quick thawing), cells from Group 2 (quick thawing and in vitro culture) predominantly exhibited enrichment in pathways related to spermatogenesis, flagellated sperm motility, cell projection organization, cilium movement, and meiotic cell cycle (Figure 6E). GO enrichment analysis of cells from Groups 3 and 4 primarily reflected pathways such as collagen fibril organization, cell adhesion, meiotic cell cycle, piRNA metabolic process, and extracellular matrix organization (Figure 6F).

In comparison with cells of Group 4, cells of Group 2 were predominantly enriched in pathways involving positive regulation of cell–substrate adhesion, positive regulation of cell differentiation, wound healing, positive regulation of axon regeneration, extracellular matrix organization, and spreading of cells (Figure 6G). It was noted that the different thawing methods after freezing may have a greater impact on the extracellular matrix during in vitro culture of testicular tissue (Figure 6H).

4.5. Discussion

4.5.1. Cryopreservation (Freezing and Thawing)

In our experiments, the protocols used for cryopreservation of testicular tissue were used [14], which were those previously used for ovarian tissue [9,12,13,15].

In our opinion, when we write about cryopreservation of ovarian and testicular tissue, the cells of both tissues can be presented as similar objects. In ovarian, as well as in testicular fragments, seven types of cell with similar intracellular structures can be differentiated. The main difference between testicular and ovarian tissues is their different density. The density of ovarian tissue is high, and testicular tissue has a loose structure. That is why, for in vitro culture of ovarian tissue fragments with a constantly stirred medium, there is no need to use encapsulation of this tissue. Whereas, due to the looseness of testicular tissue, after several days of large-volume in vitro culture with stirring of the medium, a fragment of such tissue would be dispersed into a suspension with small fragments. Therefore, we used fibrin capsules for culture of testicular tissue. However, the above differences in tissue density are insignificant in essence for cryopreservation: the cytoplasmic structures of cells of both tissues will be saturated with permeable cryoprotectants within minutes of the start of equilibration.

“Fresh” fragments (just after operation) were not an object of our experiments. This is because, for future clinical purposes, we will use only exactly cryopreserved (frozen and thawed) testicular tissues after anticancer therapy. In that way, the effectiveness of in vitro culture of “fresh” cells for our experiments is not necessary. We need to do in vitro culture of cells after cryopreservation to answer three questions regarding (1) general quality of the testicular tissue in this particular patient, (2) quality of testicular tissue in this particular patient after cryopreservation, and (3) how good is the whole cryopreservation process. The first question can be answered without in vitro culture, when a piece of testicular tissue is fixed and assessed immediately after surgery. In general, the aim of in vitro culture technology is to ensure that this technology maximally “copies” the parameters of the external (in situ) environment.

In our experiments, for thawing we used a 100 ◦C water bath. For the demonstration of this technological parameter, the following descriptions can be used: (1) when thawing in boiling water, a tampon was used to isolate the tissue fragment from the bottom of cryovial, which guaranteed no overheating; and (2) the temperature of the tissue fragment after thawing did not rise above 14 ◦C. For illustration, the cryovial felt cold 1 s after being removed from the boiling water. This technique was used because a tenet of classical cryobiology states that all types of cells and tissues, regardless of the cryopreservation method, must be thawed as quickly as possible.

4.5.2. 3D In Vitro Culture

The assessment of testicular tissue quality relies on comparing its condition before and after cryopreservation. The initial evaluation of fresh tissue offers insights into its viability post-

cryopreservation, which is crucial for determining its utility. For instance, identifying pathological changes in fresh tissue indicative of impaired spermatogenesis renders thawing unnecessary. Subsequent examination post-thaw aims to gauge the efficacy of the freezing protocol in preserving cellular functionality essential for spermatogenesis.

An illustrative demonstration of the variance in cell quality post-thawing compared to subsequent culture arises in the context of ovarian tissue vitrification, a method of cryopreservation involving direct immersion in liquid nitrogen. Initially, the evaluation of follicle quality in such tissue immediately following thawing revealed a remarkable preservation state, virtually indistinguishable from that of freshly harvested follicles. This outcome aligned with expectations, given the use of permeable cryoprotectants at high concentrations, ensuring the follicles' integrity during cryopreservation. However, within a mere six-hour culture period, nearly all follicles exhibited signs of degeneration. This striking discrepancy underscored the critical necessity for the development of dependable in vitro culture systems.

There was a previously proposed a culture system for ovarian tissue involving a large volume of culture medium with constant mixing. The efficacy of this approach with ovarian tissue fragments has been validated [20]. However, the use of the described system in relation to testicular tissue is impossible because human testicular tissue differs from ovarian tissue in density. Testicular tissue fragments tend to disintegrate into separate cell groups within 24 h in a dynamic system, impeding subsequent collection and analysis. Consequently, we devised a strategy involving the encapsulation of testicular fragments to prevent fragmentation during culture in a moving medium.

Fibrin, a substance widely present in mammalian organisms and approved for medical surgical practice by relevant regulatory organizations, such as the FDA and EU commissions, serves as a suitable encapsulating material. Its use may ensure the structural integrity of testicular tissue fragments during culture, facilitating subsequent analysis of cell quality post-culture.

The placement of tissue fragments on the periphery rather than at the center of fibrin granules, as depicted in Figure 2(A4–C4), aimed to enhance the diffusion of nutrients from the culture medium into the tissue. Examination of fibrin granules with tissue fragments after culture reveals that the tissues remain densely compacted and enveloped by a fibrin sheath (Figure 3).

After one week of in vitro culture by our method, a reduction in the volume of fibrin granule was observed. Nonetheless, tissue fragments remain ensconced in a fibrin layer (Figure 3(A5–C5)).

In our experiments (unpublished data), instances were noted where, by the end of culture, a portion of the tissue (5–20% of the surface) was devoid of the fibrin layer (Figure 3(C4–C6,C8)). However, most of the tissue remained encapsulated in fibrin, and tissue dispersion did not occur during all period of in vitro culture.

4.5.3. RNA Sequencing

The primary role of testicular tissue lies in the production of spermatozoa. Extensive sequencing investigations in humans have elucidated that testicular tissue harbors a substantial repertoire of tissue-specific genes, distinguishing it as one of the most specialized tissues within the human body [21]. Current sequencing endeavors targeting human testicular tissue predominantly center on individuals afflicted with non-obstructive azoospermia, offering novel insights into potential biomarkers and underlying mechanisms [22–25]. In our study, a comprehensive analysis of transcriptomes derived from adult testicular tissues was conducted using RNA sequencing. Specifically, it examined the impact of various thawing methods and subsequent one week of in vitro culture of testicular tissues, aiming to deepen our understanding of the effects of the described method of in vitro culture.

Differential Expressed Genes

In the present study, comparison of cells from Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) revealed a comparatively limited number of differentially expressed genes, totaling only 32. The most conspicuous upregulation was C4B_2, while SULT1A4 exhibited the most pronounced down-regulation.

C4B_2, formally known as Complement Component 4B (Chido Blood Group), Copy 2, is identified as a protein-binding gene. Its heightened expression has been observed in patients with familial primary myelofibrosis, suggesting its potential involvement in extracellular matrix formation and local inflammatory responses [26]. This observation aligns with our findings, indicating that elevated expression of C4B_2 may promote extracellular matrix development in testicular tissues during in vitro culture. Conversely, diminished expression of C4B_2 has been recognized as a risk factor for systemic lupus erythematosus, an autoimmune condition [27,28].

Turning to SULT1A4, or Sulfotransferase Family 1A Member 4, it is a pivotal enzyme in catecholamine metabolism [29]. It is noteworthy that SULT1A4 shares an important paralogous relationship with SULT1A3, and several studies have posited a potential association between

the copy number of SULT1A3/4 and neurodegenerative diseases such as Parkinson's and Alzheimer's [30]. Furthermore, recent investigations suggest that SULT1A4 may emerge as a significant target of di(2-ethylhexyl) phthalate in human granulosa cells, a compound often linked to adverse effects on female reproduction [31].

KEGG Pathways Analysis

The KEGG enrichment analysis of Groups 2 and 4 genes, characterized by a limited number of differential genes, underscored a notable enrichment in the cytokine–cytokine receptor interaction pathway. As soluble proteins that circulate outside cells, cytokines produce their effects by attaching to specific receptors located on the surface of target cells. Disruptions in cytokine–cytokine receptor interaction within the testicular milieu have been associated with various male reproductive disorders [32,33]. Additionally, cytokines play pivotal roles in the immune responses and inflammation within testicular tissue. Furthermore, comparing these two groups revealed a significant involvement of the IL-17 signaling pathway. The IL-17 cytokine family includes six members, IL-17A to F, with IL-17A exhibiting pronounced signaling pathway characteristics [34]. IL-17A is often implicated in the recruitment of inflammatory cells into the testicular interstitium, thereby compromising the integrity of the blood–testis barrier and subsequently impacting seminiferous tubule function [35]. Notably, the knockdown of IL-17A has demonstrated efficacy in mitigating testicular immune responses induced by factors such as fluoride exposure, thereby ameliorating spermatogenic damage [36]. Additionally, supplementation with probiotics has shown promise in attenuating IL-17A signaling activation driven by intestinal microbiota, consequently mitigating inflammation-induced declines in spermatozoon quality [37].

Except for cells of Groups 2 and 4, KEGG pathway analysis revealed that the lysosomal pathway exhibited the most significant changes in the remaining groups. Lysosomes, besides their role in cellular waste processing via autophagy, endocytosis, and phagocytosis pathways, play crucial roles in immune cell signaling, nutrient sensing, and metabolism [38]. During spermatogenesis, a significant proportion of testicular germ cells undergo apoptosis, which is subsequently engulfed and degraded by testicular Sertoli cells [39]. Processes such as the development of haploid germ cells, degradation of spermatozoon cytoplasm, and provision of energy for spermatozoon motility are intricately associated with autophagy [40]. The lysosomal pathway suggests extensive metabolic activity and spermatogenesis within testicular tissues during in vitro culture.

Additionally, there is significant enrichment observed in the regulation of actin cytoskeleton. The actin cytoskeleton plays pivotal roles across various stages of spermatogenesis, facilitating attachment in different cell junctions, such as basal and apical ectoplasmic specializations within the blood–testis barrier [41,42]. Basal ectoplasmic specializations regulate the transport of preleptotene spermatocytes, while the degradation of apical ectoplasmic specializations ensures the entry of fully developed spermatozoa into the lumen [43]. The dynamic renewal and homeostasis of actin are vital for spermatozoa development.

GO Terms Analysis

Cells from Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) demonstrate positive regulation of cell–substrate adhesion emerged as the top-ranked GO enrichment. Alterations in integrin attachment often drive changes in cell–substrate adhesion, consequently influencing cell migration and development. β1- integrin, along with its associated kinase integrin-linked kinase (ILK), plays pivotal roles in mediating adhesion between Sertoli cells and spermatids during spermatogenesis [44]. Furthermore, spermatid releases are mediated by a “detachment complex” containing phosphorylated FAK and α6β1-integrin [45]. Additionally, the combined GO enrichment analysis in cells from remaining groups highlighted biological processes such as spermatogenesis, cilia motility, and cell differentiation as prominently enriched, attributed to in vitro culture. GO analysis revealed distinctions between Group 3 (slow thawing) and Group 4 (slow thawing and in vitro culture) cells. In this case, cell adhesion and collagen fiber organization are more pronounced.

In our experiments, RNA sequencing of testicular tissue and testicular germ cells was performed, and somatic cells were not identified. This can only provide a general understanding of the KEGG and GO enrichment of differentially expressed genes in testicular tissue before and after in vitro culture. The adult spermatozoon maturation cycle is about 90 days but, in this study, tissue was cultured in vitro for only 7 days. The culture time is short, and only part of the stages of spermatozoon maturation can be observed.

4.6. Conclusions

Encapsulation of testicular tissue in fibrin and long-time in vitro culture with constant stirring in a large volume of culture medium can reduce the impact of thawing methods on cryopreserved testicular tissue.

Funding:

This research was funded by China Scholarship Council for Cheng Pei (No. 202208080057).

Institutional Review Board Statement:

The experiments were performed according to the Declaration of Helsinki and approved by the Bulgarian National Medical Institutional Ethics Committee (project "development of new cryopreservation methods to restore testicular function in adult and prepubertal patients with oncological diseases", approval No. 7-021/2022) and the Institutional Ethics Committee of Cologne University (protocols 01-106, 12–163, 17-427, 20-1229, code BioMSOTE).

Informed Consent Statement:

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

Data Availability Statement:

The raw RNA-seq data are accessible under BioProject: PRJNA1030294 and can be downloaded from the "Sequence read archive" on the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/1030294 accessed on 14 March 2024).

Acknowledgments:

The authors wish to express their gratitude to Mohammad Karbassian and Elvira Hilger for their technical support.

Conflicts of Interest:

Author Gohar Rahimi is employed by the company Medizinisches Versorgungszentrum AMEDES fur IVF- und Pranatalmedizin in Koln 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.

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during spermatid release from the seminiferous epithelium. J. Endocrinol. 2006, 190, 759–770. [CrossRef]

4.8. APPENDIX

Figure

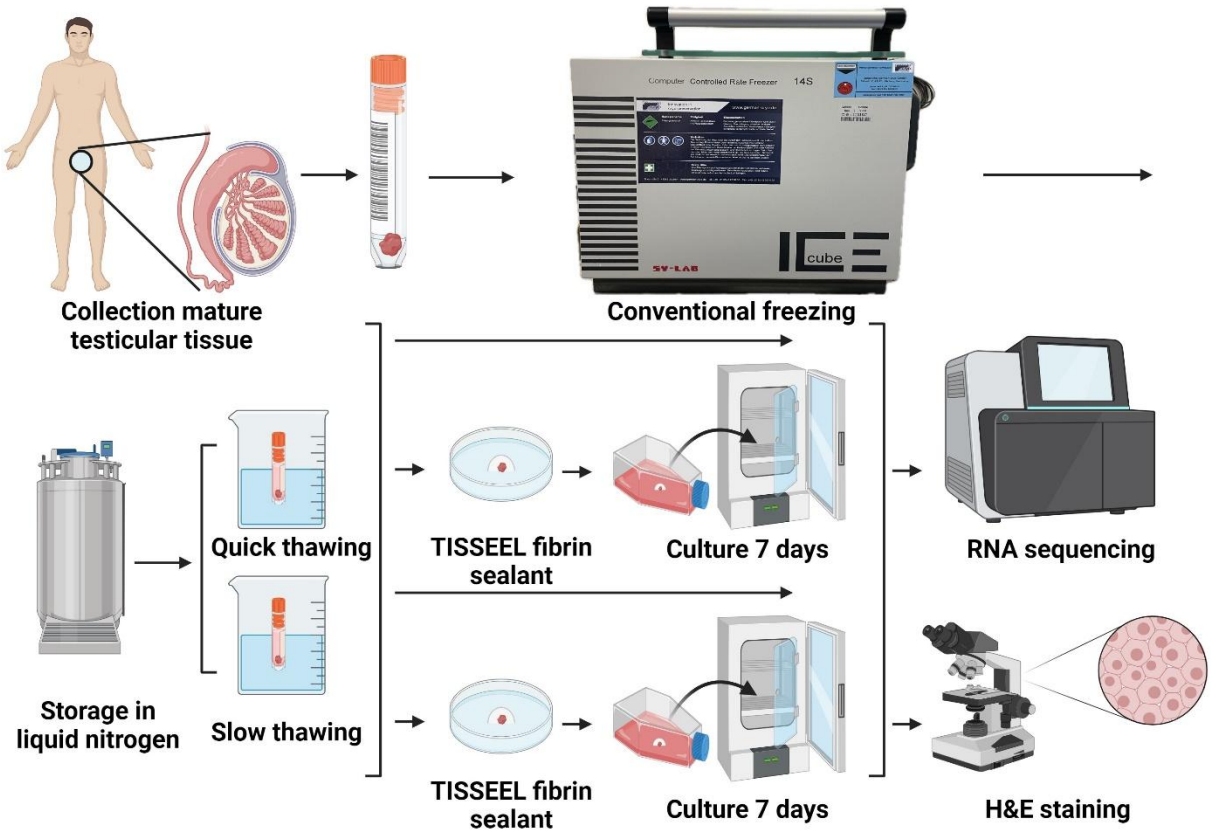

Figure 1. Design of experiments.

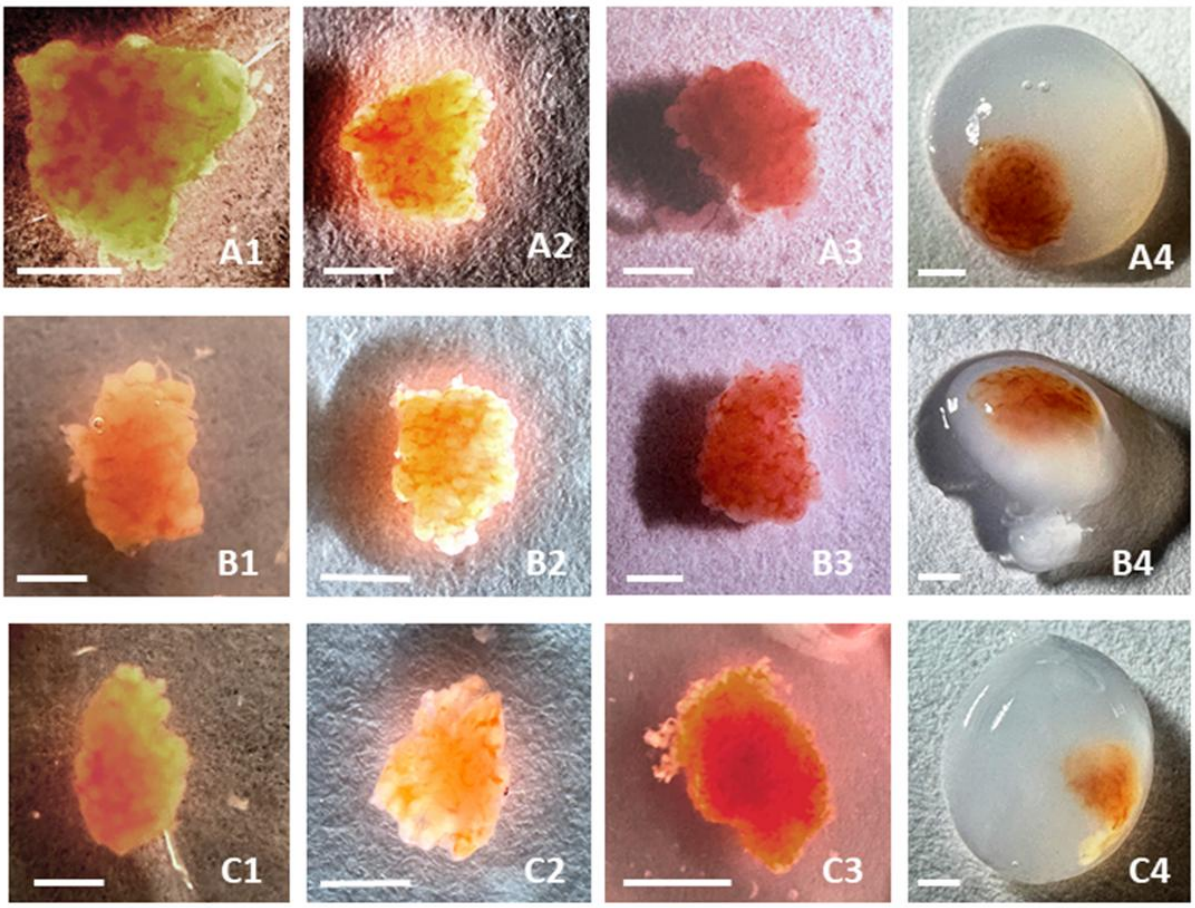

Figure 2. Cryopreserved fragments of testicular tissue from three patients. (A1–C1) Cryopreserved tissue fragments from three patients immediately after thawing (at 100 ◦C) in a freezing solution (6% dimethyl sulfoxide + 6% ethylene glycol + 0.15 M sucrose). (A2–C2) The same fragments 3 min after the beginning of removal of cryoprotectants in 0.5 M sucrose. (A3–C3) The same fragments in an isotonic solution after the end of removal of cryoprotectants (rehydration) (A4–C4) The same fragments 1 min after the beginning of formation of fibrin granules. Bar = 1.0mm.

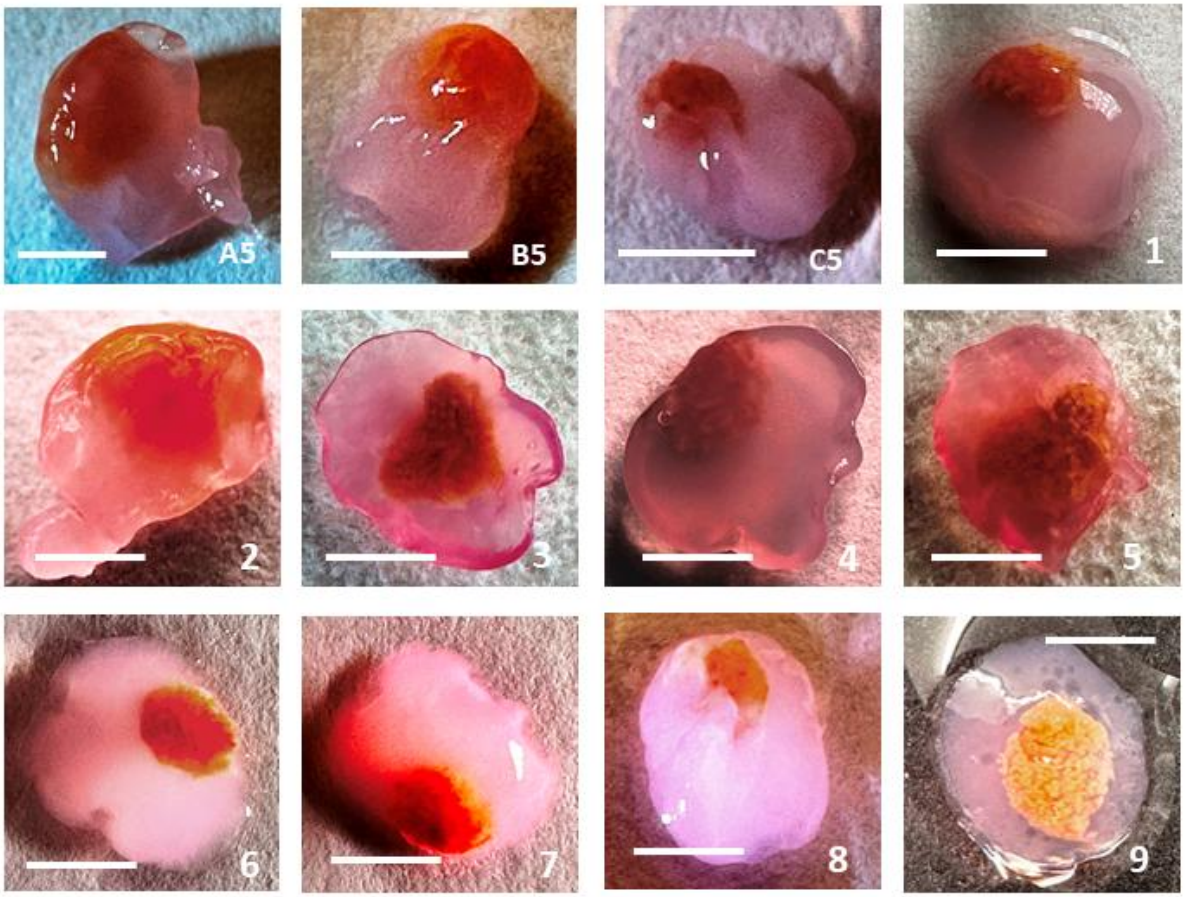

Figure 3. Cryopreserved fragments of testicular tissue sealed in fibrin granules after long-time in vitro culture. (A5–C5) Tissue fragments from three patients A, B, and C, shown in Figure 2 (1–8). Photos show the “behavior” of fibrin granules with fragments embedded using various embedding methods after long-time in vitro culture, (unpublished data). (9) The process of embedding a testicular tissue fragment in a fibrin gel: photo demonstrating the friability of the fragment and, consequently, the inevitability of its disintegration during in vitro culture. Bar = 2.0 mm**.**

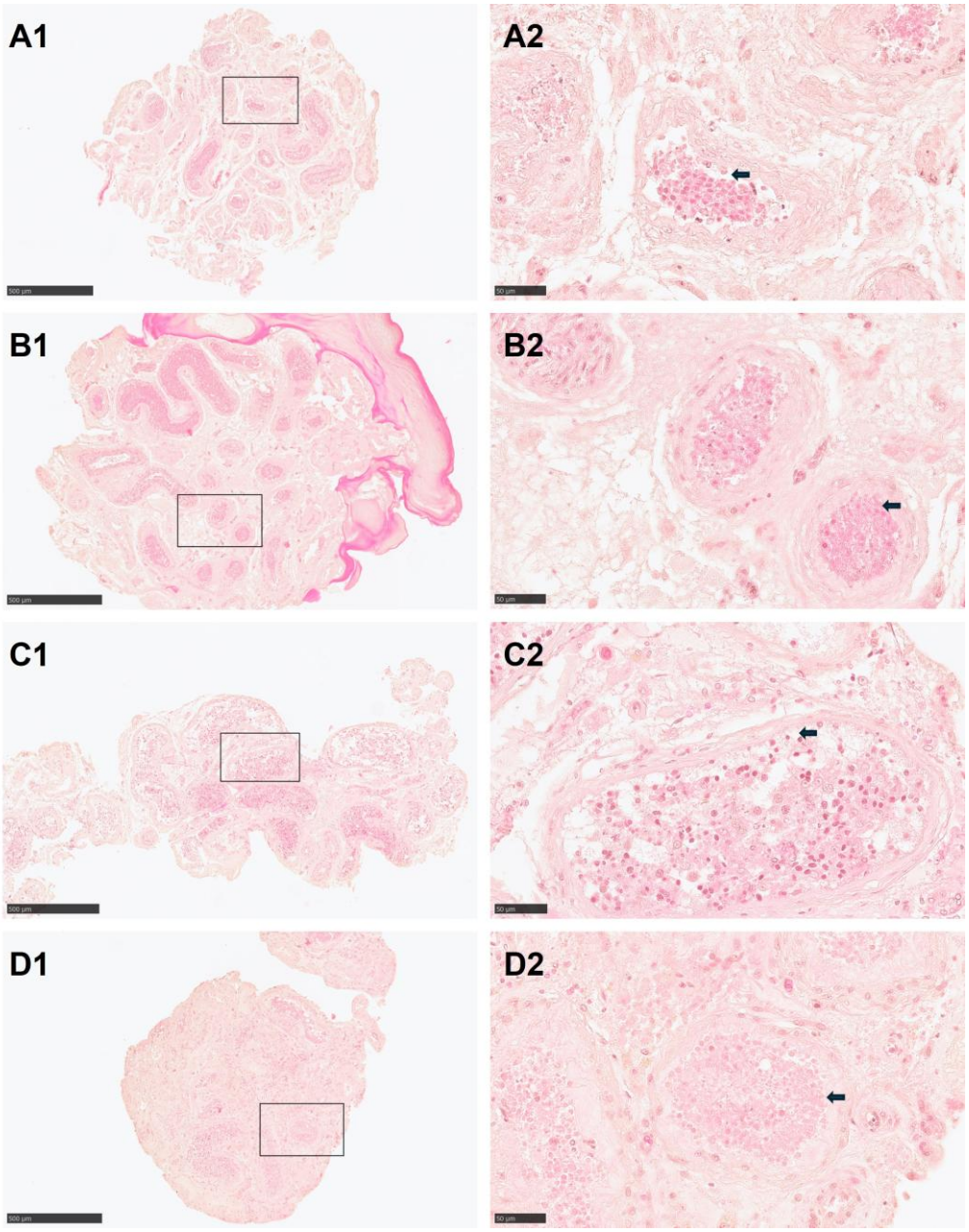

Figure 4. Hematoxylin-Eosin (HE)-staining of cryopreserved and in vitro cultured testicular tissue. (A1,A2) HE-staining of cells from Group 1 (quick thawing). (B1,B2) HE-staining of Group 2 (quick thawing and in vitro culture). (C1,C2) HE-staining of Group 3 (slow thawing). (D1,D2) HE-staining of Group 4 (slow thawing and in vitro culture). Bar for A1–D1 = 500 µm, Bar for A2–D2 = 50 µm. Black arrow indicates the space between basal membrane and cells in seminiferous tubules.

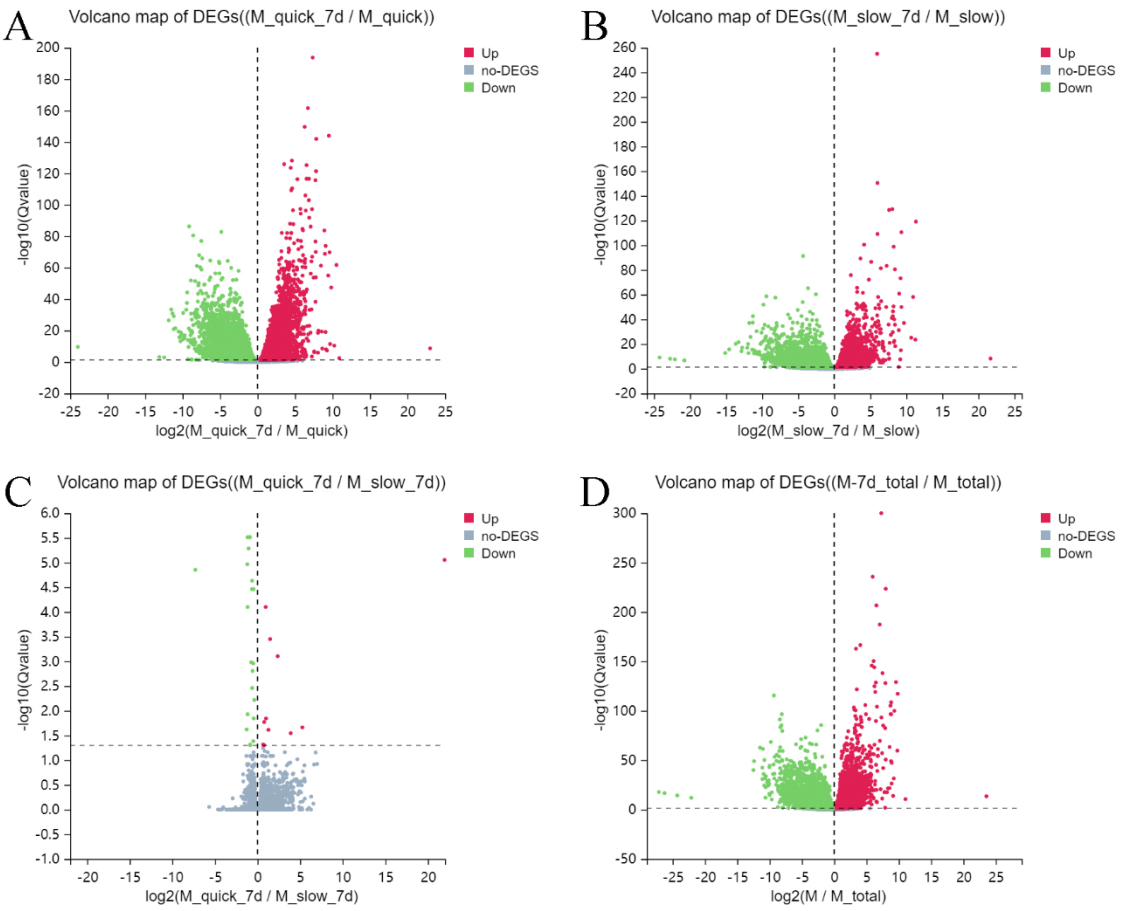

Figure 5. Volcano map showing differentially expressed genes (DEGs) between cryopreserved and in vitro cultured testicular tissue. (A) DEG volcano map: Group 2 cells (quick thawing and in vitro culture) vs. Group 1 (quick thawing). (B) DEG volcano map: Group 4 (slow thawing and in vitro culture) vs. group 3 (slow thawing). (C) DEG volcano map: Group 2 (quick thawing and in vitro culture) vs. Group 4 (slow thawing and in vitro culture). (D) DEG volcano map: Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) vs. Group 1 (quick thawing) and Group 3 (slow thawing).

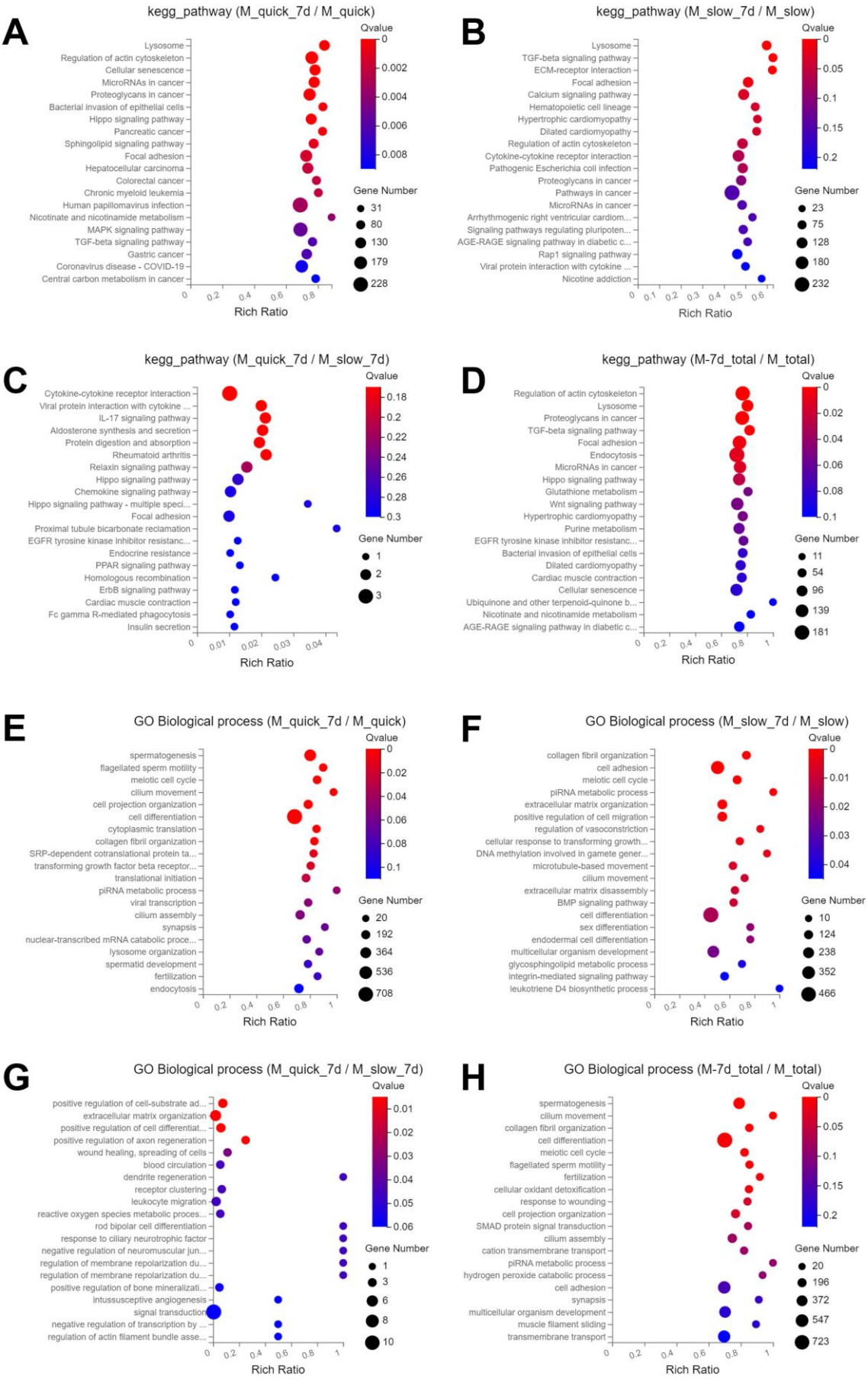
A
kegg_pathway (M_quick_7d / M_quick)
Lysosome
Regulation of actin cytoskeleton
Cellular senescence
MicroRNAs in cancer
Proteoglycans in cancer
Bacterial invasion of epithelial cells
Hippo signaling pathway
Pancreatic cancer
Sphingolipid signaling pathway
Focal adhesion
Hepatocellular carcinoma
Colorectal cancer
Chronic myeloid leukemia
Human papillomavirus infection
Nicotinate and nicotinamide metabolism
MAPK signaling pathway
TGF-beta signaling pathway
Gastric cancer
Coronavirus disease - COVID-19
Central carbon metabolism in cancer
Rich Ratio
Qvalue
Gene Number
B
kegg_pathway (M_slow_7d / M_slow)
Lysosome
TGF-beta signaling pathway
ECM-receptor interaction
Focal adhesion
Calcium signaling pathway
Hematopoietic cell lineage
Hypertrophic cardiomyopathy
Dilated cardiomyopathy
Regulation of actin cytoskeleton
Cytokine-cytokine receptor interaction
Pathogenic Escherichia coli infection
Proteoglycans in cancer
Pathways in cancer
MicroRNAs in cancer
Arrhythmogenic right ventricular cardiom...
Signaling pathways regulating pluripoten...
AGE-RAGE signaling pathway in diabetic c...
Rap1 signaling pathway
Viral protein interaction with cytokine ...
Nicotine addiction
Rich Ratio
Qvalue
Gene Number
C
kegg_pathway (M_quick_7d / M_slow_7d)
Cytokine-cytokine receptor interaction
Viral protein interaction with cytokine ...
IL-17 signaling pathway
Aldosterone synthesis and secretion
Protein digestion and absorption
Rheumatoid arthritis
Relaxin signaling pathway
Hippo signaling pathway
Chemokine signaling pathway
Hippo signaling pathway - multiple speci...
Focal adhesion
Proximal tubule bicarbonate reclamation
EGFR tyrosine kinase inhibitor resistanc...
Endocrine resistance
PPAR signaling pathway
Homologous recombination
ErbB signaling pathway
Cardiac muscle contraction
Fc gamma R-mediated phagocytosis
Insulin secretion
Rich Ratio
Qvalue
Gene Number
D
kegg_pathway (M-7d_total / M_total)
Regulation of actin cytoskeleton
Lysosome
Proteoglycans in cancer
TGF-beta signaling pathway
Focal adhesion
Endocytosis
MicroRNAs in cancer
Hippo signaling pathway
Glutathione metabolism
Wnt signaling pathway
Hypertrophic cardiomyopathy
Purine metabolism
EGFR tyrosine kinase inhibitor resistanc...
Bacterial invasion of epithelial cells
Dilated cardiomyopathy
Cardiac muscle contraction
Cellular senescence
Ubiquinone and other terpenoid-quinone b...
Nicotinate and nicotinamide metabolism
AGE-RAGE signaling pathway in diabetic c...
Rich Ratio
Qvalue
Gene Number
E
GO Biological process (M_quick_7d / M_quick)
spermatogenesis
flagellated sperm motility
meiotic cell cycle
cilium movement
cell projection organization
cell differentiation
cytoplasmic translation
collagen fibril organization
SRP-dependent cotranslational protein ta...
transforming growth factor beta receptor...
translational initiation
piRNA metabolic process
viral transcription
cilium assembly
synapsis
nuclear-transcribed mRNA catabolic proce...
lysosome organization
spermatid development
fertilization
endocytosis
Rich Ratio
Qvalue
Gene Number
F
GO Biological process (M_slow_7d / M_slow)
collagen fibril organization
cell adhesion
meiotic cell cycle
piRNA metabolic process
extracellular matrix organization
positive regulation of cell migration
regulation of vasoconstriction
cellular response to transforming growth...
DNA methylation involved in gamete gener...
microtubule-based movement
cilium movement
extracellular matrix disassembly
BMP signaling pathway
cell differentiation
sex differentiation
endodermal cell differentiation
multicellular organism development
glycosphingolipid metabolic process
integrin-mediated signaling pathway
leukotriene D4 biosynthetic process
Rich Ratio
Qvalue
Gene Number
G
GO Biological process (M_quick_7d / M_slow_7d)
positive regulation of cell-substrate ad...
extracellular matrix organization
positive regulation of cell differentiat...
positive regulation of axon regeneration
wound healing, spreading of cells
blood circulation
dendrite regeneration
receptor clustering
leukocyte migration
reactive oxygen species metabolic proces...
rod bipolar cell differentiation
response to ciliary neurotrophic factor
negative regulation of neuromuscular jun...
regulation of membrane repolarization du...
regulation of membrane repolarization du...
positive regulation of bone mineralizati...
intussusceptive angiogenesis
signal transduction
negative regulation of transcription by ...
regulation of actin filament bundle asse...
Rich Ratio
Qvalue
Gene Number
H
GO Biological process (M-7d_total / M_total)
spermatogenesis
cilium movement
collagen fibril organization
cell differentiation
meiotic cell cycle
flagellated sperm motility
fertilization
cellular oxidant detoxification
response to wounding
cell projection organization
SMAD protein signal transduction
cilium assembly
cation transmembrane transport
piRNA metabolic process
hydrogen peroxide catabolic process
cell adhesion
synapsis
multicellular organism development
muscle filament sliding
transmembrane transport
Rich Ratio
Qvalue
Gene Number

Figure 6. Bubble chart of differentially expressed genes (DEGs) displaying KEGG pathways and GO enrichment. (A) KEGG pathway chart from Group 2 (quick thawing and in vitro culture) and from Group 1 (quick thawing). (B) KEGG pathway chart of Group 4 cells (slow thawing and in vitro culture) and Group 3 cells (slow thawing). (C) KEGG pathway chart from Group 2 cells (quick thawing and in vitro culture) and from Group 4 (slow thawing and in vitro culture). (D) KEGG pathway chart of DEG in cells from Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) vs. cells of Group 1 (quick thawing) and Group 3 (slow thawing). (E) GO enrichment bubble chart for cells from Group 2 (quick thawing and in vitro culture) and Group 1 (quick thawing). (F) GO enrichment bubble chart for cells from Group 4 (slow thawing and in vitro culture) and Group 3 (slow thawing). (G) GO enrichment bubble chart for Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture). (H) GO enrichment bubble chart for Group 2 (quick thawing and in vitro culture) and Group 4 (slow thawing and in vitro culture) vs. Group 1 (quick thawing) and Group 3 (slow thawing).

5. Discussion

In recent years, fertility preservation in cancer patients, especially males undergoing treatments such as chemotherapy or radiotherapy, has received increasing attention because these treatments can adversely affect reproductive function. Cryopreservation of testicular tissue is emerging as a promising option, especially for adolescent patients who have not yet developed mature spermatozoa and adults with genetic or autoimmune diseases. Two previously published studies contributed to this research, focusing on optimizing cryopreservation protocols and long-term culture techniques to preserve male fertility.

As mentioned in the first article, there are the fewest differentially expressed genes in the comparison between group 3 and group 4. The most significantly upregulated gene is H2AC19, and the most significantly downregulated gene is GADD45B. In the comparison between group 1 and group 2, the most significantly upregulated gene is HSPA1A, which belongs to the heat shock protein family. This family is upregulated when cells are exposed to elevated temperatures or other threats to regulate the normal folding of proteins. The transcription of HSP is mainly regulated by heat shock factor 1 (HSF1) [101]. The upregulation of HSPA1A just explains why quick thawing uses 100°C water. Under physiological conditions, its intracellular expression is low, accounting for about 5%-10% of the total protein, but when exposed to stress, the expression level can be upregulated to 15%[102]. HSPA1A safeguards cells from heat stress by inhibiting ESCRT-0–mediated autophagic flux, contributing to epidermal thermoresistance[103]. HSPA1A could also improve spinal cord injury recovery in rats by inhibiting apoptosis, thereby exerting neuroprotective effects[104]. Additional studies have demonstrated that HSPA1A concentration and expression progressively decline during spermatozoa maturation and transport through the epididymis[105]. This suggests that the significant upregulation of HSPA1A during quick thawing may be beneficial to testicular tissue in reducing heat stress, inhibiting testicular cell apoptosis, and promoting sperm development.

In the comparison between group 1 and group 2, the most significantly downregulated differentially expressed gene was TKTL1. Transketolase-like-1 (TKTL1) acts as a rate-limiting enzyme in the non-oxidative phase of the pentose phosphate pathway (PPP)[106]. TKTL1 is overexpressed in malignant tumors such as cervical cancer and colorectal cancer, and high expression of TKTL1 is often associated with the invasiveness of malignant tumors[107,108]. High expression of TKTL1 often leads to enhanced glucose metabolism, thereby producing excessive lactate[109]. Therefore, inhibiting TKTL1 in malignant cells can slow the growth of malignant cells and reduce glucose consumption and lactate accumulation. In addition, studies have shown that TKTL1 may be a diagnostic marker for male azoospermia[110].

According to the comprehensive KEGG analysis, the ribosome pathway is the pathway with the most prominent changes, except for the comparison between group 3 and group 4. Ribosomes are important organelles responsible for protein synthesis in cells, and testicular tissue needs a large amount of protein synthesis because it is responsible for sperm production and hormone secretion. In groups 1 and 2, the ribosome pathway ranks first, because both are mature testicular tissues, indicating that different thawing methods will significantly affect the ribosome pathway. In the comparison between group 1 and group 3, and group 2 and group 4, the only difference between every two groups is whether the testicular tissue is mature or not, indicating that the ribosome pathway is very important for the development of testicular tissue. Liang et al. also pointed out that the KEGG enrichment analysis of men with normal sperm production and sperm production dysfunction showed that the ribosome pathway was also ranked first[111]. Recently, a new circular RNA (circRsrc1) has been discovered. The Rsrc1-161aa protein it encodes can regulate the assembly and translation of ribosomes during spermatogenesis, thereby affecting male fertility[112].

From the comprehensive analysis of GO, the SRP-dependent co-translational protein targeting the membrane pathway ranked high. This pathway is an important mechanism for targeting newly synthesized proteins in the cell membrane or endoplasmic reticulum system. When the ribosome synthesizes a new protein, if the N-terminal sequence of this protein contains a hydrophobic signal peptide, SRP will recognize and bind to this signal peptide and guide it to the SRP receptor on the cell membrane or endoplasmic reticulum membrane. This pathway prevents immature proteins from being exposed to the wrong environment[113]. The high enrichment of the SRP-dependent co-translational protein targeting the membrane pathway may be closely related to the various functions of testicular tissue. Because testicular tissue contains different cell types, such as spermatogenic cell lines and interstitial cells, there is a large demand for protein synthesis, processing, and transport. Spermatogonia and spermatocytes divide and differentiate a lot during spermatogenesis, so they need a large amount of membrane proteins and secretory proteins[114]. Leydig cells are responsible for the synthesis and secretion of hormones such as testosterone. The large enrichment of this pathway also indicates the demand of these cells for the synthesis and secretion of related enzymes and membrane proteins.

In the second article, C4B_2 was the most upregulated gene in groups 2 and 4, and SULT1A4 was the most downregulated gene. In group 2+group 4 vs group 1+group 3, the most upregulated differentially expressed gene was APP. APP stands for Amyloid precursor protein, which is expressed in a variety of tissues, especially neurons in the brain. Dominant missense

mutations of APP in the brain often cause amyloid plaques, leading to Alzheimer's disease[115]. But it is also involved in a series of normal cellular processes, such as neuronal growth, synapse formation, cell adhesion, and signal transduction. APP can dynamically regulate the activity of excitatory neurons by regulating the axon initial segment. The length of the axon's initial segment and its position in the cell can affect the probability of discharge, thereby achieving fine regulation of neuronal activity[116]. The loss of APP often leads to the loss of endothelial nitric oxide synthase protein expression and increased oxidative stress capacity[117]. APP can also regulate adipocyte mitochondrial function and obesity-related metabolism[118]. The difference between these two groups is whether they were cultured in vitro or not. The obvious upregulation of APP expression indicates that during the in vitro culture period, a large number of synapses were formed in the testicular tissue and the cell-cell adhesion increased, which is beneficial to the development of the testicular tissue.

The most significantly downregulated differentially expressed gene was HLA-DRA. HLA stands for human leukocyte antigen, and HLA-DRA is part of the human major histocompatibility complex (MHC) class II molecule, which is closely related to the immune system. The HLA-DRA gene mainly encodes the α chain of the MHC class II molecule, which forms a functional protein complex with the β chain encoded by HLA-DRB[119]. HLA-DRA often participates in the regulation of local immune responses with other immune genes. The downregulation of HLA-DRA expression indicates that after 7 days of in vitro culture, the local immune response of the testicular tissue decreased, avoiding accidental injury to testicular cells. Previous studies have also shown that HLA-DRA was found to be a bridge gene in the spermatogenesis stage through scRNA-seq[120].

KEGG enrichment analysis showed that the lysosomal pathway was the pathway with a prominent enrichment level when the testicular tissue in vitro culture group was compared with the testicular tissue non-in vitro culture group. Lysosomes are mobile, dynamic organelles that respond to intracellular and extracellular stimuli and change according to changes in the environment. Lysosomes have multiple functions, including autophagy, endocytosis, phagocytosis, and metabolic regulation[121]. During in vitro culture, testicular cells may require more resources and energy to maintain their survival and function, so they may recycle more internal resources through the lysosomal autophagy pathway (microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy), which explains the high enrichment of the lysosomal pathway [122,123]. On the other hand, during in vitro culture, the nutrients in the culture medium are sufficient, and testicular cells may more actively activate the lysosomal function, promote metabolic remodeling in testicular cells to adapt to the new environment, and repair damaged testicular cells.

GO enrichment analysis observed that the cilium movement pathway was more prominent in the rapid warming plus in vitro culture group compared with the rapid warming group alone. In testicular tissue, cilia or microcilia are closely related to sperm production and maturation. 5-Methylcytidine can effectively improve spermatogenesis by regulating the gene expression of ciliary motility[123]. Compared with the slow-warming group alone, the cell adhesion pathway was more prominent in the slow-warming plus in vitro culture group. Cell adhesion refers to the process by which cells interact with the extracellular matrix (ECM) or other cells through adhesion molecules. The main adhesion molecules involved in this process include integrins, cadherins, and selectins[124]. Different warming methods lead to different enrichments of GO, which indicates that testicular cells adopt different stress response and recovery mechanisms under different warming methods plus in vitro culture.

6. References

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