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PAPER

An *in vitro* three-dimensional (3D) testicular organoid culture system for efficient gonocyte maintenance and propagation using frozen/thawed neonatal bovine testicular tissues

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Keywords: testicular organoids, gonocytes, spermatogonial stem cells, neonatal/immature testicular tissues, cryopreservation, fertility preservation

Supplementary material for this article is available online

Abstract

Fertility preservation in prepubertal boys with cancer requires the cryopreservation of immature testicular tissues (ITTs) prior to gonadotoxic treatment. However, the limited number of germ cells in small human ITT biopsies necessitates the development of an *in vitro* culture system for germ cell expansion using frozen-thawed ITTs. Here, we generated testicular organoids for the *in vitro* maintenance and expansion of gonocytes from frozen-thawed two-week-old neonatal bovine ITTs. We investigated the effects of different cell-seeding densities, culture serums, seeding methods, and gonadotropin supplementations, on the maintenance and proliferation of enriched gonocytes. Our results demonstrated that enriched gonocytes and testicular cells from frozen-thawed neonatal ITTs could self-assemble into spheroid organoids in three days in an appropriate Matrigel-based culture environment. For the optimal formation of prepubertal testicular organoids, a seeding density of 1×10^6 cells/well is recommended over other densities. This strategy results in organoids with a mean diameter of 60.53 ± 12.12 μm; the mean number of organoids was $5.57 \pm 1.60/10^5$ μm^2 on day 11. The viability of organoids was maintained at $79.75 \pm 2.99\%$ after being frozen and thawed. Supplementing the culture medium with glial cell-derived neurotrophic factor, fibroblast growth factor 2, and leukemia inhibitory factor, increased the proportion of KI67-positive proliferating cells in organoids, elevated the expression of *C-KIT* but reduced the expression of *GFRα1* at day 28 when compared to those without hormone supplements ($p < 0.05$). In addition, supplementing the culture medium with follicle-stimulating hormone and testosterone helped to maintain a significantly higher viability ($p < 0.05$) in ITT organoids at day 28. These organoids could be cryopreserved for storage and thawed as needed. The successful generation of ITT organoids provides a valuable tool for establishing *in vitro* spermatogenesis, propagating human germ cells, investigating testicular physiology and the origin of germ cell tumors, and testing the toxicity of new drugs in future clinical applications.

1. Introduction

Childhood cancers are devastating diseases that affect children and adolescents on a global basis, with approximately 400 000 new cases diagnosed each year [1]. Fortunately, in some developed countries, the 5 year survival rate has reached 80% and continues to increase because of improvements in cancer

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therapy [2, 3]. However, the use of aggressive chemo- or radiotherapy can result in irreparable damage to pre-pubertal gonads, thus leading to hypogonadism and the reduction or depletion of germ cells; ultimately, this can lead to sterility [4, 5]. Preserving fertility in prepubertal boys facing aggressive anti-tumor therapies is a significant challenge, as unlike adult testes, they do not possess mature sperm that can be cryopreserved. At present, the only available option for these boys is to cryopreserve biopsies of immature testicular tissue (ITT). However, this strategy is associated with several challenges, and there is no definitive protocol to efficiently restore fertility from 'clinically' cryopreserved ITT stored in liquid nitrogen (LN_2). Notably, early ITT contains a limited number of germ cells, thus restricting its potential in research and clinical scenarios. Therefore, there is an urgent need to develop an efficient *in vitro* culture system for germ cell propagation and maintenance that can bridge this gap and enable targeted research on the use of cryopreserved ITT to restore fertility.

Gonocytes, also known as prospermatogonia, are the fundamental germ cells that exist in the early stages of testis [6, 7]. These germ cells reside at the center of the seminiferous cords and travel progressively to the basement membrane and continue their proliferative activity in early prepuberty. These biological shifts are essential for fertility, and any abnormality in gonocyte development can lead to infertility. The regulation of germ cell proliferation and differentiation in the germ cell niche relies upon cell interactions and specific growth factors [8]. During prepuberty, the serum levels of follicle-stimulating hormone (FSH), luteinizing hormone, and testosterone remain low, but increase during puberty. The serum levels of anti-Müllerian hormone (AMH) declines and inhibin B increase. Collectively, these changes lead to testicular growth. In addition, growth factors such as glial cell-derived neurotrophic factor (GDNF), fibroblast growth factors (FGFs), and retinoic acid, are crucial for spermatogonial stem cell (SSC) development [9–13]. The proliferation of SSCs can be enhanced during *in vitro* culture by leukemia inhibitory factor (LIF) and GDNF [14]. Therefore, to support the *in vitro* culture of ITT and re-establish an appropriate microenvironment for the maintenance or differentiation of gonocytes, it is crucial to devise an appropriate supplementation strategy that includes different hormones.

Testicular organoids, intricate three-dimensional structures that emulate the *in vivo* microenvironment, exhibit the potential to restore fertility by facilitating the cultivation and manipulation of germ cells [15–18]. Previous investigations have generated testicular organoids from the prepubertal testicular tissues of several species, including prepubertal mice at 7 and 10 d post-partum (dpp) [19, 20], fresh 5-dpp and 8-dpp rats [21], fresh or frozen 4–7 dpp pigs [22, 23], human embryonic gonads [24], and human prepubertal testicular tissues (6 month-old and 5 year-old) [20]. Due to the scarcity and value of human testicular tissue, we used bovine ITTs in this study as a research model; these tissues underwent the same rigorous and standardized clinical cryopreservation processes as human tissues at the Oxford Cell and Tissue Biobank (OCTB). Holstein bulls commence spermatogenesis at 20 weeks-of-age and complete this process by 32 weeks-of-age [25], thus providing a lengthy window of prepubertal/pubertal development for research investigations. Furthermore, bovine testes have a very similar structure to human testes and their testicular microenvironment more closely resembles that of human testes than that of mice or rats, thus making them a valuable model for studying developmental processes in the prepubertal testis. The development of *in vitro* culture systems for testicular cells is a promising approach that allows for the observation of cells under controlled conditions by applying bioengineering techniques. To the best of our knowledge, only one prior study employed fresh pubertal (7–10 months) bovine testicular cells to produce testicular organoids [26]. In the OCTB, the youngest individuals who have undergone cryopreservation of their testicular tissues were merely a few months old. At present, there is no established early prepubertal testicular organoid culture system capable of supporting early-stage germ cells, such as gonocytes, from frozen-thawed ITTs in the bovine model that were processed and frozen with a clinically viable technique. Therefore, we aimed to address this research gap by developing an early prepubertal testicular organoid culture system using ITTs that were processed and cryopreserved using an established clinical protocol.

The objective of this study was to establish an efficient prepubertal testicular organoid culture system using frozen-thawed early-stage bovine ITTs to maintain and propagate gonocytes/SSCs *in vitro*. We also investigated the combination of various hormones to develop an effective hormone supplementation strategy for the *in vitro* culture of prepubertal testicular organoids. By utilizing a clinical cryopreservation program, we demonstrate the potential for the future *in vitro* maintenance and propagation of SSCs and other clinical applications using cryopreserved human ITTs.

2. Materials and methods

2.1. Acquisition and cryopreservation of testicular tissue

Six bovine testes were obtained from three two-week-old neonatal Holstein calves (*Bos taurus*) at Tockenham Corner abattoir (Swindon, UK).

All testes were transferred on ice to the laboratory in transfer medium containing sterile Hanks' Balanced Salt Solution (Sigma-Aldrich, Missouri, US) supplemented with 2% penicillin-streptomycin (Sigma-Aldrich), 0.1 M sucrose (Sigma-Aldrich), and 10 mg ml^{-1} of bovine serum albumin (BSA, Thermo Fisher Scientific, Massachusetts, US). ITTs were then dissected into fragments ($3 \times 3 \times 3$ mm^3 in size) and processed using a standard clinical testicular tissue cryopreservation operating procedure that is used routinely for human tissue in the Oxford Cell and Tissue Biobank (OCTB; Oxford, UK) [27]; cryopreservation was performed in a computer-controlled freezer (Ice Cube 14 S, SY-LAB, Purkersdorf, Austria). Tissue fragments were then stored in LN_2 for future analysis. When thawing, vials were removed from LN_2 and immersed in a 37 °C water bath for 2 min; this was followed by three 5 min washes with cold transport medium.

2.2. Histology and immunohistochemistry of ITTs

The testicular tissue fragments were fixed in 4% formalin (Sigma-Aldrich, UK) overnight and embedded in paraffin wax. Serial sections (5 μm thick) were then prepared from the embedded tissues using a microtome (Leica, Germany).

For hematoxylin and eosin (H&E) staining, tissue sections were first deparaffinized and rehydrated and then stained in haematoxylin solution (Sigma-Aldrich, UK) for 30 s, followed by eosin solution for 1 min.

For immunohistochemical staining, we first inactivated endogenous peroxidases by applying 0.3% of H_2O_2. Non-specific binding sites were then blocked with goat serum blocking solution for 30 min at room temperature. Primary antibodies were then incubated with ITT sections overnight at 4 °C. Anti-PGP9.5 primary antibody (1:100 dilution; Abcam, Cambridge, UK) was used as a marker for gonocytes/SSCs and anti-vimentin primary antibody (1:200; Santa Cruz Biotechnology, CA, USA) was used as a marker for Sertoli cells (table S1); although vimentin is not an exclusive marker for Sertoli cells, it has been used effectively in previous related studies [28–31]. The following morning, sections were washed with phosphate buffered saline (PBS) and then incubated with the secondary antibody provided in the VectaStain™ ABC kit (Vector Laboratories, Burlingame, CA, USA). Subsequently, signals were visualized by 3,3′-diaminobenzidine; this was followed by counterstaining with hematoxylin. In our experimental approach, we included negative controls which were performed in parallel experiments using only secondary antibodies. The stained sections were then evaluated for morphological appearance using a Nikon microscope (Japan).

2.3. Isolation and enrichment of primary neonatal testicular cells

After thawing, tissues were dissociated into single-cell suspensions using a two-step enzyme digestion procedure according to a previously published method [32] with some modifications. In brief, tissues were mechanically disaggregated and cut using needles and a scalpel; then, the fragment was incubated in the first digestion solution (table S2) at 34 °C for 30 min with shaking followed by a second incubation in the second digestion solution at 34 °C for 30 min with shaking. Dissociated testicular cells were collected and suspended in culture medium containing 5% Percoll density gradient (Hunter Scientific, Saffron Walden, UK); this was carefully placed onto layers of a 30%–40% Percoll density gradient followed by centrifugation for 25 min at 1000 × g and 34 °C. Testicular cells within and between different Percoll density phases were then collected and washed thoroughly for further processing.

2.4. Preparation, culture, and 3D organoid culture

Enriched testicular cells were suspended in culture medium, mixed with Matrigel on ice at a ratio of 1:1, and then carefully dispensed as 50 μl droplets on pre-warmed plates followed by incubation at 34 °C for 30 min to polymerize the gel (figure 1(A)). Culture medium was then added to cover the Matrigel dome after polymerization. Cell densities of 1×10^5 cells, 1×10^6 cells, and 1×10^7 cells per Matrigel droplet were used in the experiment. The basic culture medium was Minimum Essential Medium-alpha (MEM-α) supplemented with 10% knockout serum replacement (KSR) and other supplements (table S3). In some experiments, the organoid culture medium was supplemented with growth factors, including FSH (10 IU/L; Sigma-Aldrich), testosterone (10^{-6} M; Sigma-Aldrich), GDNF (20 ng mL^{-1}; Sigma-Aldrich), FGF2 (2 ng ml^{-1}; Thermo Fisher Scientific), and LIF (100 ng ml^{-1}; Thermo Fisher Scientific). Organoids were passaged every 10–14 d. The medium was changed every two days, and the sizes of the testicular cell spheroids were recorded.

2.5. Cell viability

Cell viability was assessed using a live/dead cell imaging kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Images were acquired using a fluorescence microscope and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5.1. Cryopreservation and recovery of testicular organoids

All cryovials containing the organoids and 1 ml of cryoprotectant were maintained at 4 °C in a Mr. Frosty freezing container (Thermo Fisher Scientific) for 30 min and then transferred to

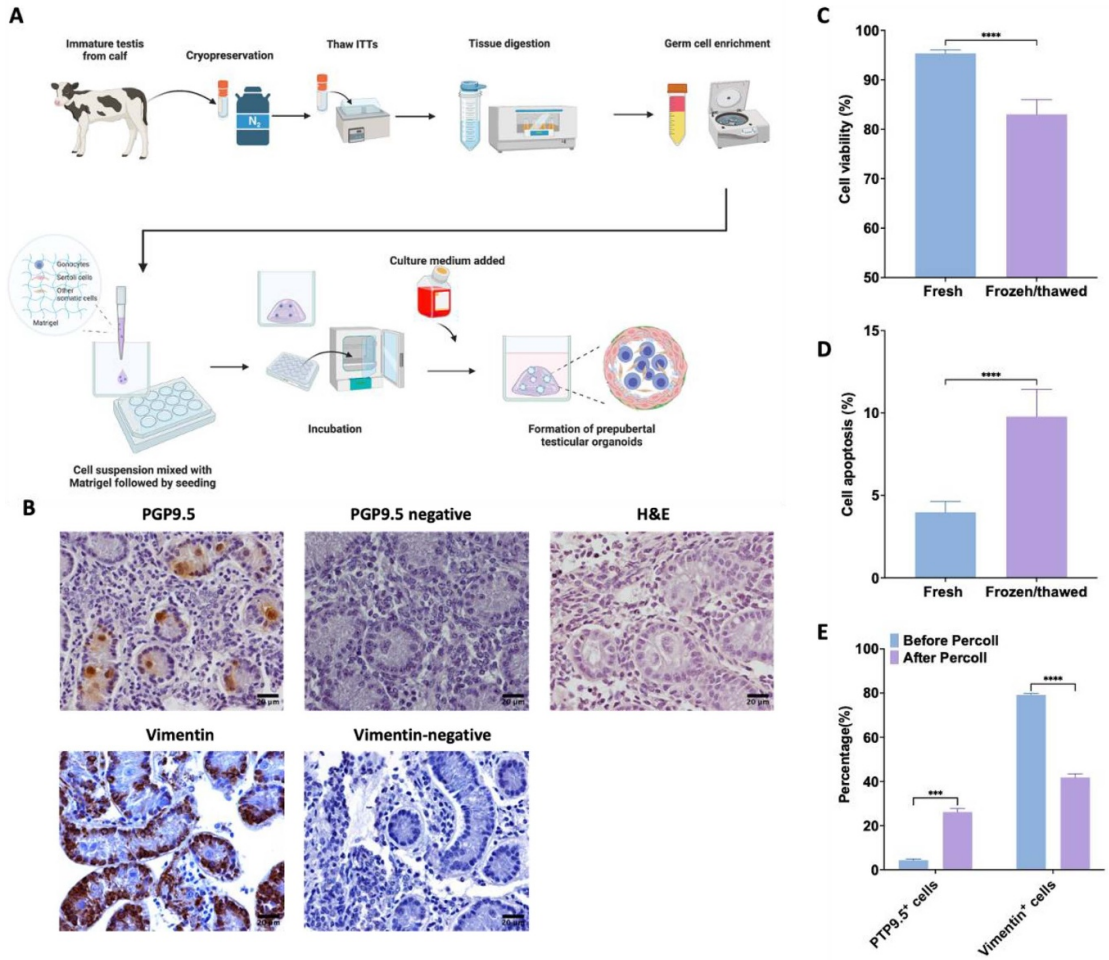

Figure 1. Overall view of the establishment of the prepubertal testicular organoid culture system and testicular cell preparation. (A) Frozen and thawed ITTs were digested and enriched using a Percoll density gradient. The cells were then suspended in culture medium and mixed with Matrigel on ice. The resulting mixture was seeded as small droplets and placed into an incubator. Once the Matrigel became gel-like, culture medium was added. After a few days of *in vitro* culture, testicular organoids were formed inside the Matrigel dome. The figure was created with BioRender.com. (B) Representative immunohistochemistry and staining with PGP9.5 and Vimentin antibody and H&E (hematoxylin and eosin) staining of frozen/thawed two-week-old bovine testes. Scale bar = 20 μm. Cell viability (C) and apoptosis (D) were measured before and after the frozen/thawed procedure. (E) The proportions of germ cells (PGP9.5 positive) and Sertoli cells (vimentin positive) were assessed before and after Percoll gradient enrichment. Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

a −80 °C freezer overnight followed by long-term storage in LN_2. When thawing, organoids were removed from the LN_2 tank and immediately transferred to a 34 °C water bath for 2 min followed by two washes in culture medium.

2.6. Immunocytochemical analysis of organoids

The immunocytochemical staining of testicular organoids was optimized based on a generalized protocol for all 3D organoids [33]. Anti-PGP9.5 antibody and anti-PLZF antibody were used to identify neonatal gonocytes/SSCs while an anti-vimentin antibody was used to identify Sertoli cells through immunocytochemical staining (table S1). An anti-KI67 antibody were used to stain proliferating cells. In brief, testicular organoids were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 (v/v) (Sigma-Aldrich) for 15 min, and then blocked using 1% BSA for 1 h at room temperature. The organoids were then incubated with primary antibodies at optimized dilutions (table S1) for 24 h at 4 °C. The organoids were then washed three times followed by incubation with secondary antibodies for 24 h at 4 °C. After three washes, the organoids were mounted using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). We employed confocal microscopy to capture Z-stack images of the entire organoid. The representative images presented in our study were selected from the middle sections of the organoids, thus allowing us to provide a representative view of their internal composition. Images were analyzed by Volocity software (PerkinElmer, Waltham, MA, USA) and ImageJ.

2.7. RNA extraction and qRT-PCR

Total RNA was extracted from testicular organoids using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. In brief, testicular organoids were lysed and homogenized, followed by incubation with a mixture of chloroform and isoamyl alcohol; the colorless aqueous layer containing RNA was then collected, placed in a spin cartridge, allowed to bind to the membrane, and washed. Purified RNA was stored at −80 °C for subsequent analysis.

cDNA was synthesized from 100 ng of RNA using the SuperScript™ IV One-Step RT-PCR Kit (Thermo Fisher Scientific) and a reverse transcription thermal cycler program. qRT-PCR assays were performed in 96-well plates with a QuantStudio™ 3 system (Applied Biosystems, Foster City, CA, USA). In each reaction, a total volume of 20 µl per well was used, containing 4 µl of diluted cDNA templates, 10 µl of Fast SYBR Green Master Mix (Thermo Fisher Scientific), 1 µl each of 10 µM forward and reverse primers, and 4 µl of DNase-/RNase-free water. Samples were run in triplicate along with a non-template control. *β-ACTIN*, which has been identified and validated as a stable gene for qRT-PCR in bovine testes, was used as a housekeeping gene. Seven selected genes were targeted in this research (table S4), including *PGP9.5, PLZF, GFRα1, C-KIT, STRA8, AMH, and STAR*. Data were analyzed by Microsoft Excel™ (Microsoft, Redmond, WA, USA) and the $2^{-\Delta\Delta Ct}$ method [34].

2.8. Statistical analyses

The Shapiro–Wilk test was used to test the normality of quantitative data. Data with a normal distribution are presented as a mean ± standard deviation, whereas data with a non-normal distribution are presented as a median (25% percentile, 75% percentile). GraphPad Prism 9.3.0 software (GraphPad Software, San Diego, CA, USA) was used to create graphs and perform tests for statistical significance. Statistical differences between groups were analyzed by the student's *t*-test or by analysis of variance. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Cryopreservation and the dissociation of ITTs

Figure 1(A) depicts an overall view of the establishment of the bovine prepubertal testicular organoid culture system. Bovine ITTs were cryopreserved using a standard clinical testicular tissue cryopreservation program in the OCTB to mimic the processing and condition of human ITTs. Subsequently, the ITTs were digested, enriched, and seeded in an appropriate 3D culture system.

Histological analysis of the frozen/thawed tissues revealed well-preserved seminiferous cords in bovine ITTs. Immunohistochemistry staining successfully identified germ cells (PGP9.5 positive) and Sertoli cells (Vimentin positive) (figure 1(B)). To assess the quality of the frozen/thawed ITTs, cell viability and apoptosis were measured before and after the cryopreservation procedure. After the cryopreservation program, the cell viability of the frozen/thawed tissues was 83.02 ± 2.96% (figure 1(C)); the proportion of cellular apoptosis was 9.78 ± 1.67% (figure 1(D)). A Percoll gradient density was then utilized to enrich isolated testicular cells, resulting in a significant increase in the proportion of germ cells to 26.13 ± 2.73% (figure 1(E)). The Percoll-enriched cells were subsequently used for the generation of prepubertal testicular organoids.

3.2. Effect of cell-seeding density on the formation of immature testicular organoids

To optimize the cell-seeding density in 3D culture and investigate the effects of cell density on the formation of organoids in 3D culture, we first tested three cell densities: 1×10^5 cells/well, 1×10^6 cells/well, and 1×10^7 cells/well, in Matrigel droplets (50 µl) in 24-well plates (figure 2(A)). The formation of testicular organoids was observed at 24–48 h after seeding in Matrigel. The presence of germ cells and Sertoli cells in the testicular organoids was confirmed by immunocytochemical staining for established germ cell markers (PGP9.5 and PLZF) and a Sertoli cell marker (vimentin), respectively (figure 2(B)). In 3D testicular organoids, we observed structures with Sertoli cells surrounding the exterior of the organoids, while germ cells were present at the centers or on one side. The presence of Sertoli cells and Leydig cells in testicular organoids was confirmed by analyzing the gene expression of *AMH* and *STAR* separately by qRT-PCR (as shown in figure 2(E)) during the culture period. The expression levels of *AMH* and *STAR* remained relatively stable and did not show significant changes throughout the 14 day period of culture.

We measured the diameters of the organoids as an indicator of their relative size. There were no significant differences in the diameter of organoids on day 2 when compared across the three groups (figure 2(C)). The diameters of organoids on day 2 in the 1×10^5 cells/well group, 1×10^6 cells/well group, and the 1×10^7 cells/well group, were 32.79 ± 6.26 µm, 37.14 ± 10.86 µm, and 41.79 ± 10.04 µm, respectively. These diameter measurements were similar to the diameter of 2 week-old neonatal bovine seminiferous cords, which ranged from approximately 30–50 µm, as determined by H&E staining of intact ITTs obtained from the same calves. On day 11, the mean diameter of organoids in the 1×10^7 cells/well group was 68.87 ± 17.30 µm; this was significantly greater than the diameter of organoids in the 1×10^5 cells/well

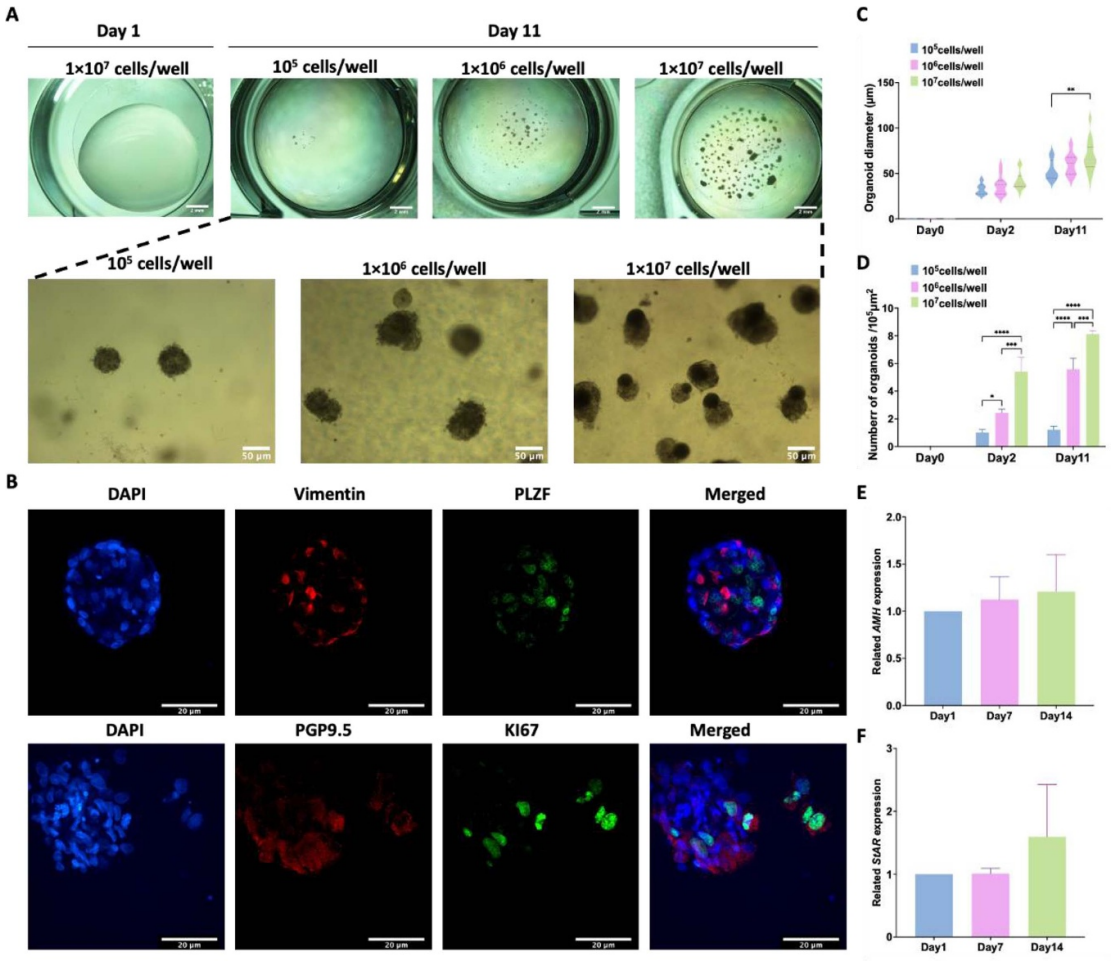

Figure 2. The formation of organoids from enriched testicular cells seeded at different initial seeding densities in three-dimensional (3D) Matrigel culture. (A) Representative brightfield images of testicular organoids after 1 d of culture of initially seeding cells at a density of 1×10^7 cells/well. The Matrigel dome was clear and transparent. After 11 d of culture, testicular organoids were formed at densities of 1×10^5 cells/well, 1×10^6 cells/well, and 1×10^7 cells/well cell-seeding densities. Scale bar in top images = 2 mm. Scale bar in bottom images = 50 μm. (B) Representative immunofluorescence images of testicular organoids showing the expression of germ cell markers (PLZF and PGP9.5), vimentin, and KI67. Scale bar = 20 μm. (C) The diameters of organoids were measured for each group with different seeding densities. (D) The number of organoids per 10^5 μm^2 was counted. Three biological replicates were performed ($n = 3$). Expression levels of AMH (E), a marker of immature Sertoli cells, and *STAR* (F), a marker of Leydig cells throughout the 14 day period of culture. Data are shown as the mean ± standard deviation. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

group (53.54 ± 11.02 μm; $p < 0.05$). The mean diameter of organoids in the 1×10^6 cells/well group was 60.53 ± 12.12 μm.

In addition, we quantified and analyzed the number of organoids formed at varying seeding densities. The mean number of organoids in the 1×10^6 cells/well group was 2.43 ± 0.60/10^5 μm^2 on day 2 and 5.57 ± 1.60/10^5 μm^2 on day 11; this was significantly greater than the organoids in the 1×10^5 cells/well group on day 2 and day 11 ($p < 0.05$, figure 2(D)). The mean number of organoids in the 1×10^7 cells/well group was 5.41 ± 1.79/10^5 μm^2 on day 2 and 8.11 ± 0.55/10^5 μm^2 on day 11; these values were significantly higher than the other two groups on day 2 and day 11, respectively ($p < 0.05$).

Considering the limited number of testicular cells available in neonatal testicular tissues, and the inconsistency of the size and number of organoids, we selected a density of 1×10^6 cells/well for testicular organoid generation in subsequent experiments.

3.3. Effect of serum-free culture medium on organoids

Next, we investigated the effects of serum or serum replacements by culturing 3D testicular organoids in culture medium supplemented with 10% fetal bovine serum (FBS), 5% KSR, 10% KSR, or 15% KSR. As with our previous 2D culture results [35], no cellular aggregation was observed in cells cultured with MEM-α + 10% FBS (figure 3(A)). However, organoid formation was observed in culture medium containing low KSR supplementation (MEM-α + 5% KSR) (figure 3(B)). Testicular cells cultured in MEM-α + 10% KSR and MEM-α + 15% KSR culture medium formed organoids in our *in vitro* 3D system with a similar rate and size (figures 3(C) and (D)).

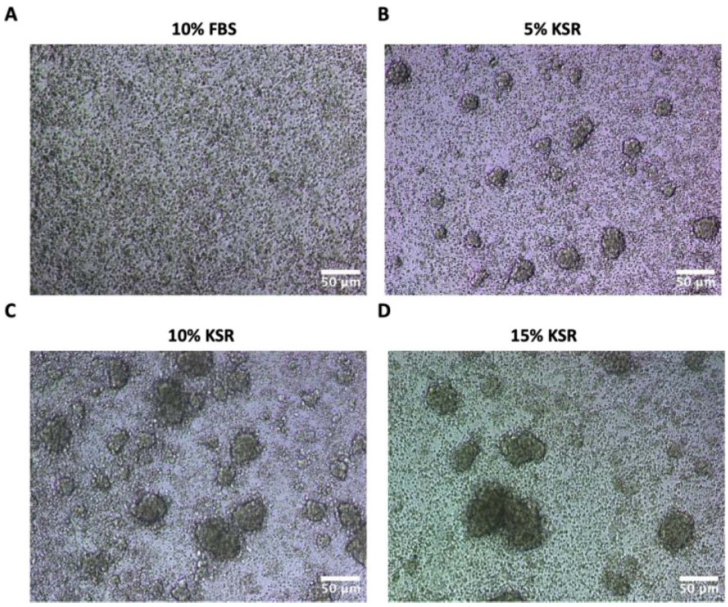

Figure 3. Formation of organoids cultured in culture medium supplemented with serum or serum replacement. (A) No organoids were formed in MEM-α medium with 10% fetal bovine serum (FBS) in the three-dimensional (3D) Matrigel culture system. However, organoids were formed after 1 d of culture in a 3D Matrigel culture system using MEM-α medium supplemented with 5% (B), 10% (C), or 15% (D) knockout serum replacement (KSR). Scale bar = 50 μm. Three biological replicates were performed ($n = 3$).

3.4. Effect of cell-seeding methods with Matrigel

Next, we investigated and compared the effects of three different cell seeding methods on the formation of testicular organoids, including 'mixed-seeding', 'inside-seeding' and 'on top-seeding'. In the 'mixed-seeding' group, culture medium with suspended cells was mixed with the same volume of Matrigel at a 1:1 ratio to form an organoid mixture. Then, one droplet of the mixture was carefully seeded into a plate and incubated at 34 °C for 30 min to allow the dome to polymerize; this was followed by the addition of 1 mL of culture medium to each well. In the 'inside-seeding' group and the 'on top-seeding' group, a drop of a Matrigel:medium (1:1) mixture was incubated at 34 °C for 10 min for polymerization before the cell suspension was injected into the middle of, or on top of, the Matrigel dome, respectively. This was followed by a 20 min incubation at 34 °C before adding 1 mL of culture medium to each well. We found that the organoids formed at a faster rate with clearer boundaries during the first 72 h in the 'inside-seeding' group and the 'on top-seeding' group (figure 4).

3.5. Cryopreservation and recovery of immature testicular organoids

To investigate the feasibility of cryopreserving testicular organoids over the long-term for clinical and practical purposes, six cryoprotectant agents (CPA1–CPA6) were used to cryopreserve organoids. Testicular organoids were collected *in situ* and divided into six groups before being cryopreserved in different CPAs using a Mr. Frosty freezing container. After 60 d of storage in LN_2, organoids were thawed, and cell viability was determined by live/dead cell imaging. Cell viability in the post-cryopreserved organoids was significantly lower than that in fresh testicular organoids ($p < 0.05$; figure 5). Comparison among the CPA groups indicated that cell viability in the CPA3 group was 79.75 ± 2.99% and that this was significantly higher than the 73.33 ± 0.58% cell viability in the CPA5 group ($p < 0.05$). Of all the cryopreservation groups tested, the CPA3 group exhibited the highest cell viability and was, therefore, selected for subsequent experiments.

3.6. Effects of growth factors on the *in vitro* maintenance of organoids

To generate a microenvironment for gonocytes, we next investigated the effects of different growth factor combinations within testicular organoid cell culture by evaluating the expression levels of selected genes (figure 6(A)). In the control group, the basic culture medium consisted of MEM-α, 10% KSR, and basic additives. In the GDNF + FGF2 + LIF (GFL) group, GDNF, FGF2, and LIF were added to the basic culture medium; our aim was to promote self-renewal/proliferation. In the FSH + testosterone (FT) group, FSH and testosterone were added to the basic culture medium to promote differentiation. Cell viability was assessed by live/dead assay on days 1, 7, 14, and 28 in the three groups (figures 6(B) and (C)). On day 28 of culture, the cell viability was significantly higher in the FT group when compared to the control group ($p < 0.05$). Live/dead cell staining of 192 testicular

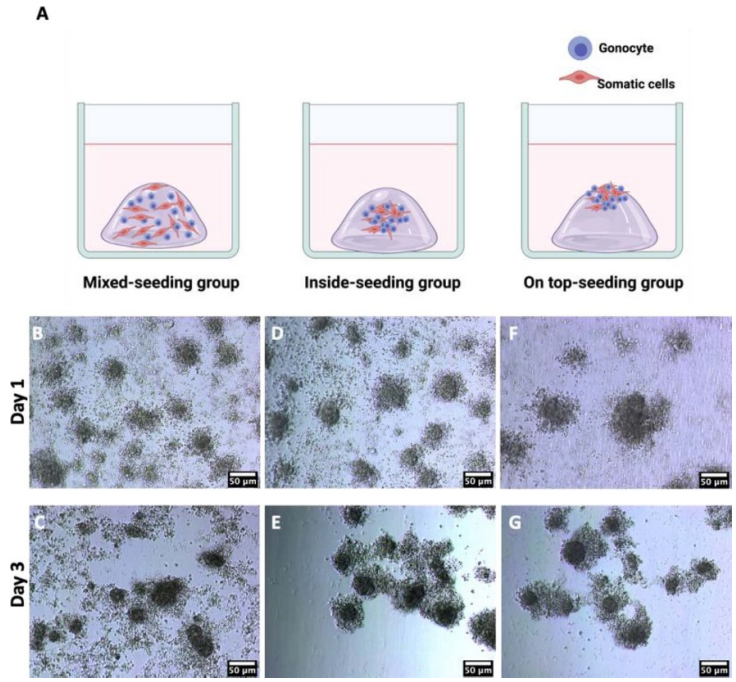

Figure 4. Formation of testicular organoids in three cell-seeding methods in a 3D Matrigel culture system. (A) Three methods of cell seeding. (B) and (C) Testicular cell suspensions were mixed with the same volume of Matrigel (mixed-seeding group) before cell seeding and cultured for 1 d (B) and 3 d (C). (D) and (E) Cells were injected into the Matrigel after incubation for 10 min (inside-seeding group) and were cultured for 1 d (E) and 3 d (E). (F) and (G) Cells were gently seeded on top of the Matrigel after incubation for 10 min (on top-seeding group) and cultured for 1 d (F) and 3 d (G). Scale bar = 50 μm. Three biological replicates were performed ($n = 3$). Figure created with BioRender.com.

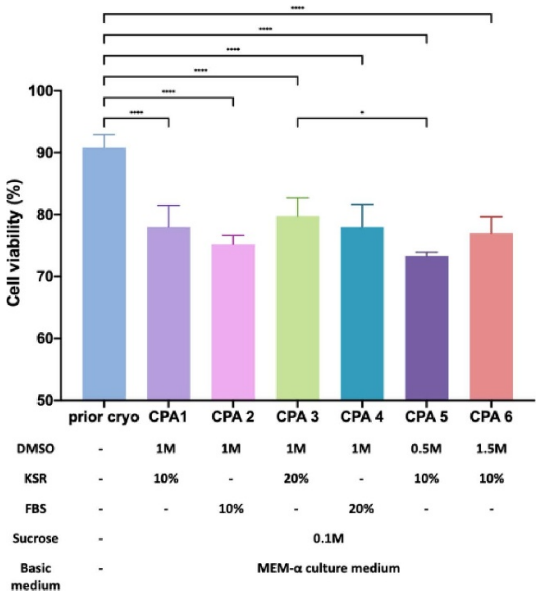

Figure 5. Cell viability prior to and after prepubertal testicular organoid cryopreservation and the composition of six different cryoprotectant agents (CPAs). Cell viability was evaluated by live/dead staining. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; KSR, knockout serum replacement.

organoids results showed that dead cells began to appear at the center of the organoids. During culture, the organoids in the three groups showed similar round shapes but were of various sizes (figures 6(D) and (E)). The mean size of the organoids was evaluated on days 3, 7, and 14 after cell seeding. Significant differences were evident between the three groups on days 7 and 14 ($p < 0.05$). The mean size of organoids on day 7 was 505.4 ± 51.44 μm^2, 593.8 ± 47.17 μm^{2,} and 380.0 ± 40.25 μm^2 in the control, GFL, and FT groups, respectively. On day 14, the mean size of the organoids was 618.7 ± 39.3 μm^2, 1030.00 ± 116.5 μm^{2,} and 768.4 ± 78.49 μm^2 in the control, GFL, and FT groups, respectively.

In addition, the organoids were analyzed by immunocytochemistry to identify the germ cells and proliferating cells present in the organoids. Proliferating cells (KI67-positive) were present in significantly higher numbers in organoids on day 28 in the GFL group ($p < 0.05$, figures 6(F) and (G)), but fewer proliferating cells were detected in the control group. No significant changes were observed in the proportion (%) of PGP9.5-positive cells in organoids during culture over a 28 d period (figure 6(H)). The expression levels of selected genes were measured by qRT-PCR (figure 6(I)). The expression levels of *GFRα1*, a gonocyte marker, had decreased significantly by 76% in the control and 78% in the GFL groups on day 28 when compared to the levels on day 1 ($p < 0.05$). There was no significant change in the expression of *PGP9.5,* an established marker for SSCs. The gene expression levels of *PLZF* were significantly reduced on day 14 in all three groups

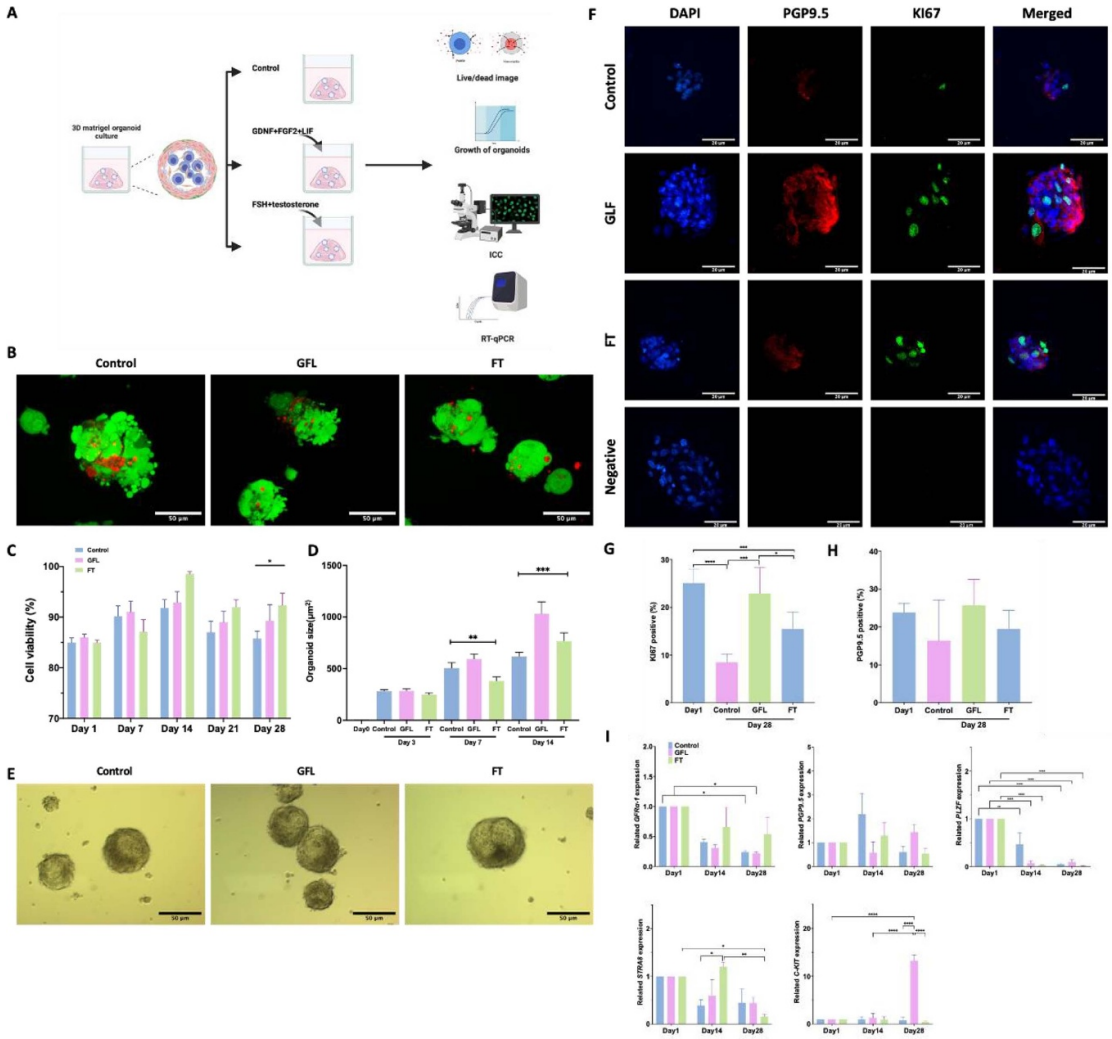

Figure 6. Effects of different growth factors on the *in vitro* growth of testicular organoids. (A) According to experimental design, organoids were divided into the following three groups: a control group, a GDNF + FGF2 + LIF (GFL) group, and a FSH + testosterone (FT) group. All organoids were cultured for 28 d in a 3D Matrigel culture system. Figure created with BioRender.com (B) Representative viability images of organoids on day 28 of culture. (C) Representative brightfield images of organoids on day 28 of culture. D) Viability of cells in organoids was assessed on days 1, 7, 14, 21, and 28. Data are presented as the mean ± SEM. (E) Changes in organoid size during the first 14 d after cell seeding in the three groups. Scale bar = 50 μm. (F) Representative immunocytochemical images of gonocytes in the three groups on day 28. Negative: negative control staining performed with only the secondary antibody. Positive staining for the PGP9.5 germ cell marker (red) and KI67 proliferation cell marker (green). (G) Percentage of KI67-positive cells on day1 and day 28. (H) The proportion (%) of PGP9.5-positive cells on day 1 and day 28. (I) The effects of different hormones on organoids were evaluated by comparing the expression levels of germ cell-related genes, including *GFRα1, UCH-L1, PLZF*, and *STRA8*, as well as the *c-KIT* spermatogenesis-related gene. GDNF, glial cell-derived neurotrophic factor; FGF2, fibroblast growth factor 2; LIF, leukemia inhibitory factor; FSH, follicle-stimulating hormone. Three biological replicates were performed ($n = 3$). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

when compared to the levels on day 1 ($p < 0.05$); these levels remained low on day 28. The expression of *C-KIT,* a spermatogenesis-related gene, was significantly increased on day 28 in the GFL group when compared to the GFL group on day 1 and on day 14, the control group, and the FT group on day 28 ($p < 0.05$).

4. Discussion

In the present study, we developed an *in vitro* 3D Matrigel-based system and optimized culture conditions utilizing enriched frozen/thawed neonatal bovine testicular cells for the maintenance of gonocytes/SSCs. We also compared effects of different combination of hormones in 3D culture. We processed neonatal bovine ITTs with the same clinical cryopreservation program used for human tissues in order to facilitate potential translation to human ITTs in the future. In the presence of a suitable 3D scaffold and the requisite biochemical components, testicular cells demonstrated the capacity to autonomously organize themselves into organoids. These organoids establish an *in vitro* microenvironment with Sertoli cells surrounding the outside and germ cells located in the middle or only one side of the organoids.

Due to the low proportion of SSCs in ITT, we used enriched gonocytes/SSCs in our culture system and provided sufficient nourishment and support. Our present findings showed that even at a relatively lower cell-seeding density (e.g. 10^5 cells/well), isolated testicular cells from immature bovine testis could successfully self-assemble into organoids, which could potentially be applied to small young human ITT biopsies in the clinic. We also found that a higher cell density of 1×10^7 cells/well increased the diameter and number of organoids formed in each well. During culture, dead cells and cellular apoptosis were easier to identify in the center of organoids with larger diameters when applying live/dead cell imaging and TUNEL staining; this may be attributed to the lack of space, nutrients, or oxygen for cells located at the center of organoids. Therefore, to better control the size and quality of organoids, a moderate cell-seeding density of 1×10^6 cells/well was considered as the optimal initial cell-seeding density for generating neonatal bovine testis organoids. In previously developed testicular culture systems, the initial cell-seeding densities varied widely, including 2.8×10^5 cells/well, 5×10^5 cells/well, 6×10^5 cells/well, and 2×10^6 cells/well [20, 36, 37].

The biophysical features of Matrigel are dependent on the concentration of the polymer incorporated. This factor is critical for the formation of organoids from different tissues. Matrigel is more fluid-like at low temperatures, and solidifies over time, reaching a consistent state after a few hours in an incubator. In the present study, we tested different seeding procedures and found that cells migrated more rapidly when seeded on the top or inside of the Matrigel after incubation for 10 min when compared to cells suspended in culture medium and Matrigel mixture prior to incubation. These results may be explained by the observation that when seeded inside or on top of the Matrigel, the cells were suspended within the culture medium and were more able to move rapidly, thus making it easier for them to bind to each other and form cell clusters. Mixtures with 25% or higher concentrations of Matrigel gradually become solid-like within 3 h and then remain stable [38]. Therefore, during the 10 min incubation at the beginning of our experiments, the Matrigel dome was predominantly liquid-like; thus, the testicular cells injected within or on top of the Matrigel were able to migrate freely due to the lower polymer concentration. It is possible that within the central region of the Matrigel dome, the polymer concentrations are lower, thus allowing cells in this area to form organoids more rapidly. Further studies now need to establish whether the factors secreted by cells or from the ECM can guide cellular migration and cause changes in cellular morphology.

The efficient and effective long-term organoid storage may accelerate the development of organoid technology and clinical translation. The cryopreservation of intact organoids instead of dissociated organoids is recommended for intestinal organoids due to better cell recovery [39]. Based on the findings of the present study, we suggest the use of 1 M DMSO, 20% KSR, and 0.1 M sucrose in the cryoprotectant used to cryopreserve prepubertal testicular organoids. Our results showed that over 70% of cells in the frozen/thawed organoids remained viable, and their viability remained high in subsequent cultures. The cryopreservation of 3D organoids enhances our potential to produce and store testicular organoids from ITTs on a large scale to allow organoids to become an accessible *in vitro* model for clinical use or drug toxicity tests.

Developing an *in vitro* germ cell culture system poses challenges for cell survival, maintenance, proliferation, and maturation. A range of growth factors are commonly used for the short- and long-term culture of SSCs. Only limited research has been performed on gonocyte cultures using growth factors. One reason for this may be that gonocytes only exist for a short time after birth; furthermore, we know little about the mechanisms involved in their functionality. Our results demonstrated that the use of a culture medium supplemented with GDNF, FGF2, and LIF, resulted in the generation of larger organoids; this was consistent with increased SSC expansion reported in a previous study [40]. The increased size of germ cell colonies/organoids may be the result of different cytoskeletal organizations due to GDNF and FGF2, which separately promote flat colony formation and colony clumping [40]. Our results also showed that the combination of GDNF, FGF2 and LIF maintain a higher proportion of proliferating cells (KI67-positive), including proliferating gonocytes. This might relate to the regulation of GDNF for the self-renewal of SSCs *via* the GDNF/RET/GFRA1 signaling pathways [10, 41]. Previous studies have shown that the GDNF/RET/GFRA1 signaling pathway activates the PI3K/AKT intercellular signaling pathway, thereby influencing the expression of *BCL6B, ETV5, LHX1, ID4*, and *POU3F1* transcription factors which have been demonstrated to promote SSC self-renewal [42, 43]. FGF2, which is produced and released by mammalian Sertoli cells, drives *in vitro* SSC self-renewal by upregulating the expression levels of *ETV5, BCL6B*, and *LHX1* genes *via* MAP2K1 activation [44]. SSC proliferation is also regulated by autocrine activation of the PI3K/AKT and MAPK/ERK pathways by FGF2 [45]. Research has also shown that FGF2 is involved in the expansion of SSCs and the formation of germ cell colonies [40]. LIF has been shown to maintain the pluripotency of mouse embryonic stem cells and the self-renewal of SSCs. GDNF, FGF2, and LIF have all been used for

the long-term maintenance of porcine SSCs *in vitro* for up to 1 month in 2D cultures [46]; these findings concur with our current results.

With regards to gene expression levels, the combination of GDNF, FGF2, and LIF maintained the expression levels of the *UCH-L1* and *STRA8* germ cell markers but reduced expression levels of the *GFRα1* gonocyte marker, thus indicating the transformation from gonocytes to undifferentiated SSCs. Interestingly, a significant increase in the gene expression levels of *C-KIT*, which is associated with spermatogenesis, was observed on day 28 in organoids cultured with GDNF, FGF2, and LIF. However, a previous study suggested that c-kit is associated with the migration of neonatal rat gonocytes to the basement membrane within one week after birth and is associated with the pseudopods that appear on gonocytes during migration [47]. It is possible that the expression of *C-KIT* is suppressed and remains at a relatively low level in the testes of neonatal calves prior to the migration of gonocytes. When migration starts, the expression of the *C-KIT* gene increases and supports gonocyte migration *in vitro*. In the present study, immunocytochemical staining with the KI67 proliferation cell marker detected a higher number of proliferating germ cells (UCH-L1-positive) in organoids cultured with GDNF, FGF2, and LIF, thus indicating the transformation of gonocytes to proliferating SSCs.

When added to the culture medium, FSH and testosterone resulted in a higher cell viability on day 28 of testicular organoid culture. The expression levels of the *STRA8* gene increased and then decreased during the 28 d of culture, showing that no advanced differentiation of germ cells was detected during culture. Culture with FSH and testosterone resulted in a larger mean diameter of the organoids on day 28, as well as a higher number of Ki-67-positive cells compared to control group, thus indicating that FSH and testosterone might also support the proliferation of cells in organoids. During the postnatal development of Holstein bulls, there is a notable increase in the concentration of FSH which reaches maximal levels at 2 months and 5 months of age, respectively [48]. Previous research has shown that the FSH signaling pathway may be associated with gonocyte transformation [49]. Further studies might need to investigate the different combinations of multiple growth factors as well as the mechanisms underlying the influence of gonadotrophins on the development of gonocytes.

The present study had several limitations that need to be considered. First, we mainly optimized culture conditions for 28 d to grow and maintain organoids *in vitro*. Further studies need to investigate longer periods of culture. Second, we did not investigate *in vitro* spermatogenesis from gonocytes to sperm or the potential maturation of other somatic cells in the present study. Thirdly, further studies are warranted to investigate the potential to utilize animal models to advance our understanding of human ITTs. Finally, in this study, we utilized a restricted set of markers to identify various cell types within prepubertal testicular organoids. To achieve a more comprehensive understanding of cellular development in cultured organoids, additional markers should be incorporated, particularly those specific to different developmental stages of germ cells or somatic cells within the stem cell niche. Furthermore, it is necessary to consider that the development of SSCs and their associated niches from post-birth to peri-puberty in calves and humans remains significantly underexplored. Addressing this substantial gap in our understanding requires further investigations to unravel the intricate processes involved during this critical developmental phase. Consequently, while limited by a restricted set of markers, the present study represents a significant milestone in advancing our understanding of testicular organoid generation. It is important that future studies fully characterize the cellular composition of such organoids by applying immunocytochemical and molecular sequencing approaches.

5. Conclusion

Herein, we report the establishment of an *in vitro* 3D neonatal testicular organoid culture system by investigating a series of culture conditions that affect the formation, storage, and development of gonocyte organoids, including cell-seeding density, serum replacement supplement, Matrigel ratio, organoid cryopreservation, and the presence of growth factors. Gonocytes in organoids were cultured *in vitro* for up to 28 d. During culture, we observed Sertoli cells surrounding the outside while germ cells were located in the middle or only one side of the organoids. We also observed proliferating germ cells on day 28 of culture. We also proved that these neonatal testicular organoids can be efficiently cryopreserved for further use with good cell viability and minimal cellular apoptosis. In addition, GDNF, FGF2, and LIF supplementation maintained a high proportion of proliferating cells, while promoting the transformation of gonocytes into SSCs. FSH and testosterone were found to be beneficial for maintaining the viability and proliferation of cells in organoids. Importantly, 3D neonatal testicular organoids represent a novel tool for *in vitro* studies of germ cell development, cellular interactions, endocrinology, and toxicity.

Our findings indicate that we successfully developed an *in vitro* immature testicular organoid culture system which could possibly be applied in many future scenarios, including (1) human germ cell/Sertoli cell/SSC niche development during

prepuberty and peri-puberty; (2) human germ cell number expansion for future research or clinical application; (3) *in vitro* spermatogenesis and fertility restoration for prepubertal boys with cancer; and (4) drug toxicity screening for personalized medicine.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Conflict of interest

The authors confirm that there are no known conflicts of interest related to this publication.

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