Determining the optimal time interval between sample acquisition and cryopreservation when processing immature testicular tissue to preserve fertility

Original research

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Abstract

The cryopreservation of immature testicular tissue (ITT) prior to gonadotoxic therapy is crucial for fertility preservation in prepubertal boys with cancer. However, the optimal holding time between tissue collection and cryopreservation has yet to be elucidated. Using the bovine model, we investigated four holding times (1, 6, 24, and 48 hours) for ITTs before cryopreservation. Biopsies from two-week-old calves were stored in transport medium and cryopreserved following a standard slow-freezing clinical protocol. Thawed samples were then assessed for viability, morphology, and gene expression by haematoxylin and eosin (H&E) staining, immunohistochemistry and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Analysis failed to identify any significant changes in cell viability when compared between the different groups. Sertoli (Vimentin+) and proliferating cells (Ki67+) were well-preserved. The expression of genes related to germ cells, spermatogenesis (*STRA8, PLZF, GFRα-1, C-KIT, THY1, UCHL-1, NANOG, OCT-4, CREM*), and apoptosis (*HSP70-2*) remained stable over 48 hours. However, seminiferous cord detachment increased significantly in the 48-hour group ($p<0.05$), with associated cord and SSC shrinkage. Collectively, our analyses indicate that bovine ITTs can be stored for up to 48 hours prior to cryopreservation with no impact on cell viability and the expression levels of key genes. However, to preserve the morphology of frozen-thawed tissue, the ideal processing time would be within 24 hours. Testicular tissues obtained from patients for fertility preservation often need to be transported over long distances to be cryopreserved in specialist centres. Our findings highlight the importance of determining optimal tissue transport times to ensure tissue quality in cryopreservation.

Keywords: Fertility preservation; delayed processing; immature testicular tissues; bovine;

cryopreservation

Introduction

The field of cancer therapy and research has advanced significantly over recent decades, leading to a considerable rise in the five-year net survival rate of children diagnosed with cancer. The survival rate for childhood cancer patients has now exceeded 80% in some developed countries [26; 32; 44]. However, despite these achievements, it is crucial to consider the potential risks associated with cancer treatments, particularly the adverse effects on male reproduction. Research has shown that exposure to cytotoxic cancer treatments can induce the loss of germ cells, thus leading to infertility in adult survivors of childhood cancer [39; 42]. It is important to note that as the survival rate continues to improve, the risk of infertility caused by cancer or gonadotoxic cancer treatments may become more prevalent. While the cryopreservation of semen and assisted reproductive technology (ART) can help to restore fertility in adult males, such technology cannot be used for prepubertal boys as these patients have not yet started to produce mature sperm. Therefore, the first step towards establishing clinical strategies to preserve fertility in prepubertal boys is to cryopreserve samples of immature testicular tissue (ITT) before initiating cancer therapy. To address this issue, a number of centres around the world have established specialized cryopreservation programs to cryopreserve ITT in anticipation of future strategies for fertility restoration in prepubertal boys [20; 29; 33; 43]. Although there is currently no practical clinical strategy for restoring fertility in prepubertal boys from frozen ITT, a landmark study recently demonstrated that cryopreserved ITT from rhesus monkeys were capable of undergoing spermatogenesis when thawed and generated healthy offspring following autologous transplantation [17]; these

achievements present us with a highly promising foundation for the future restoration of fertility in humans.

In the UK, the Oxford Reproductive Tissue Cryopreservation Programme (ORTCP) was established in 2008 to provide tissue cryopreservation options for children with cancer. The ORTCP has third-party agreements with most principal cancer treatment centres across England, Wales, and Ireland [25]. The programme procures ITTs, transports them to the Oxford Cell and Tissue Bank (OCTB), and processes the ITTs with a standardized testicular tissue cryopreservation programme. However, the distance between a third-party centre and the Oxford laboratory is highly variable. The Nordic Centre for Fertility Preservation (Nordfertil) is known to transport human ITTs by air over long distances; for example, Iceland to Stockholm. In other countries, however, it is possible that human ITTs may need to be stored for longer periods in order for them to be transported to a laboratory for cryopreservation, thus leading to delayed processing times. In addition to the transit time needed to transport tissues from the patient to the laboratory, sometimes tissues need to be stored for longer time periods due to the availability of cryopreservation machines or other forms of clinical coordination. In some cases, the overall delayed processing time (the summation of transit time and holding time, representing the time elapsed from tissue procurement to cryopreservation) could be up to 48 hours. Appropriate transport conditions and the storage period prior to cryopreservation could be essential for ensuring the ITT remains viable for subsequent clinical use. Previous studies have investigated the effect of short-term storage on fresh testicular tissues from human adults [15; 16]; however, no prior study has investigated the effects of delayed processing on frozen-thawed ITT. A major concern is that the pre-exposure of ITT to a period of prolonged cold ischaemia may enhance the harmful effects of cryopreservation on the health and integrity of ITT. Therefore, it is necessary to investigate the effects of delayed tissue processing time on the viability of cryopreserved ITT for future clinical use.

Given the relative lack of immature human testicular tissue available for research, we used immature testicular tissues from a bovine model. The bovine model offers distinct advantages for research. Firstly, its extended prepubertal phase, in comparison to mouse or rat models, provides an advantageous context for the study of prepubertal testis. In Holstein bulls, the maturation of testicular development is initially slow during the period from birth to 20 weeks [46]. Specifically, at one month-of-age, the tubules in calves are small and do not have a lumen [36]. This mirrors the absence of a distinct lumen in human seminiferous tubules, which typically become observable at approximately 3 to 4 years-of-age [6]. Notably, within the first 20 weeks after birth, the seminiferous epithelium in young calves consists of SSCs and Sertoli cells, while advanced spermatids are not yet present; this provides a lengthy window of prepubertal development for research. These first 20-weeks of calf development could be compared to birth to approximately 8 years-of-age in terms of the prepubertal human testis. The onset of spermatogenesis commences around 20-weeks of postnatal life and full spermatogenic cycles are established by around 32-weeks [11], thus providing a pubertal time for research studies. Secondly, bulls exhibit a relatively low efficiency of spermatogenesis, generating approximately 12 x 10^6 sperm per gram of testicular tissue daily, a rate akin to that found in humans (4 to 6 x 10^6 sperm per gram of testicular tissue daily). Thirdly, the duration of spermatogenesis in bulls, approximately 61 days, closely mirrors the 74-day duration observed in humans [2; 22].

We processed bovine ITT using the standard collection, transport and cryopreservation protocol that is used clinically at the ORTCP for human tissues. We used this strategy to investigate the specific effects of various processing times (1 hour, 6 hours, 24 hours, and 48 hours) on the viability of ITT using a clinical protocol for future fertility preservation. The findings of our study may help to improve the efficiency and efficacy of clinical fertility

preservation programs and thus facilitate the future restoration of infertility in the survivors of childhood cancer.

Methods

Collection and preparation of testicular tissues

ITTs were collected from six testes obtained from three 2-week-old Holstein calves (*Bos taurus*) at Tockenham Corner abattoir (Swindon, UK), which would be otherwise discarded. For the ethical aspect, Home Office licensing is not required in this case. Upon acquisition, ITT biopsies were immediately transferred to the laboratory on ice using transfer medium. The transfer medium was composed of Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, USA) supplemented with 2% penicillin-streptomycin (Pen–Strep, Sigma-Aldrich, St. Louis, USA), 0.1 M sucrose (Sigma-Aldrich, St. Louis, USA), and 10 mg/mL bovine serum albumin (BSA; Thermo Fisher Scientific, Waltham, USA). Upon arrival at the laboratory, the adipose tissue and tunica albuginea were removed, and longitudinal incisions were created to expose the testicular parenchyma. Then, the testicular tissues were dissected into $3 \times 3 \times 3$ mm^3 fragments using a surgical scalpel (Swann Morton, Sheffield, UK) for further processing (**Figure 1B**).

To simulate the transport of human tissue, tissues were kept in transfer medium at 4°C for varying durations before cryopreservation: 1 hour (the control group), 6 hours (a short transportation time), 24 hours (standard transportation time), and 48 hours (a long transportation time) (Figure 1A). These four processing times refer to the time elapsed from the moment the tissues were retrieved from the slaughterhouse and the commencement of the cryopreservation program. Tissue fragments were then cryopreserved and stored in liquid

nitrogen (LN_2), mirroring the delays that could occur clinically during fertility preservation. We utilized the standard testicular cryopreservation programme used routinely by the ORTCP, followed by viability and apoptosis assessments post-thaw. Histological, immunohistochemistry, and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analyses were also conducted.

Cryopreservation and thawing of bovine testicular tissue fragments

Testicular tissue fragments were processed following the standard clinical protocol for human testicular tissue cryopreservation as established by the ORTCP [25]. Relevant adjustments were made for bovine tissues; this involved the replacement of human serum albumin (HSA) with bovine serum albumin to ensure species compatibility. In brief, tissue fragments were equilibrated in a pre-made cryoprotectant agent (CPA) containing HBSS supplemented with 1.5 M Me_2SO (DMSO; Sigma-Aldrich, St. Louis, USA), 0.1 M sucrose, and 10 mg/mL bovine serum albumin (BSA). Cryopreservation was performed using a controlled-rate freezer (Ice Cube 14S, SY-LAB, Neupurkersdorf, Austria) with a specific testicular tissue cryopreservation program that featured different cooling rates and holding times. The program comprised of a start temperature of 4°C, followed by cooling rate I (-1°C/min to 0°C); a 5 min hold period; cooling rate II (-0.5°C/minute to -8°C); a 5 min hold period; automatic seeding of the cryovials followed by tissue soaking for 15 min; cooling rate III (-0.5°C/minute to -40°C), a 10 min hold period; cooling rate IV (-7°C/minute to -70°C) and finally, cooling rate V (-10°C/minute to -140°C). After freezing, tissues were stored in the vapour phase of LN_2.

For thawing, the cryovials containing the testicular tissue fragments were rapidly thawed in a 37°C water bath for 2 – 3 minutes. Subsequently, the tissue fragments were washed with three

183 laboratory-made thawing solutions featuring a decreasing gradient of Me_2SO (0.75 M, 0.375
184 M, and 0 M, separately), a sucrose concentration of 0.1 M, and 10 mg/mL BSA in HBSS
185 medium, for 5 minutes each at 4°C.

186 ## Histology and immunohistochemistry

187 The testicular tissue fragments were fixed overnight in 4% formalin (Sigma-Aldrich, St. Louis,
188 USA) before being embedded in paraffin wax. The embedding procedure was carried out by
189 the histology facility at the Kennedy Institute of Rheumatology at the University of Oxford.
190 Serial sections (5 μm in thickness) were obtained from the embedded tissues using a microtome
191 (Leica, Wetzlar, Germany). At least three sections from different parts of each of the embedded
192 tissues were used for haematoxylin and eosin (H&E) staining or immunohistochemical
193 staining. Both H&E staining and immunohistochemical staining were performed after the
194 sections were dewaxed with xylene and rehydrated in a series of decreasing ethanol
195 concentrations.

196 The morphology of the testicular tissue was evaluated by H&E staining. For H&E staining,
197 slides were incubated in haematoxylin solution (Sigma-Aldrich, St. Louis, USA) for 30
198 seconds, followed by eosin solution (Sigma-Aldrich, St. Louis, USA) for 1 minute, and then
199 washed in running water for 10 minutes. Microscopic images of the stained tissue sections were
200 captured using a Nikon microscope (Tokyo, Japan), and the analysis of seminiferous tubule
201 detachment from the basement membrane was conducted as described below. The histology of
202 the ITTs was classified as Grades 1, 2, or 3, based on the main morphological patterns,
203 structural integrity, and architecture, considering the attachment of tubular cells to the basement
204 membrane (**Figure 2A**). Seminiferous tubules were considered intact when tubular cells
205 adhered to the basement membrane. Tubules were graded as follows: Grade 1 (full cellular
206 adhesion or < 30% detachment from the basement membrane), Grade 2 (partial detachment

(30% to 70%) from the basement membrane), and Grade 3 (> 70% detachment from the basement membrane). The assessment of % detachment involved measuring the extent of the detached length encircling the tubule with respect to the entire basement of the tubule. Nine distinct tissue sections, sourced from three individual animals, were analysed for each of the four time points. This resulted in three biological replicates at each time point, culminating in a total of 36 slides. Within these slides, a total count of 1683 tubules was performed. The measurements and counting were performed in a blinded approach by an experienced examiner.

Immunohistochemical staining was performed as follows. First, the sectioned tissues were deparaffinized and rehydrated, and then endogenous peroxidase was inactivated by treatment with 0.3% H_2O_2. Non-specific binding sites were then blocked with goat serum blocking solution for 30 minutes 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 all gonocytes/spermatogonial stem cells (SSCs), anti-vimentin primary antibody (1:200; Santa Cruz Biotechnology, CA, USA) was used as a marker for Sertoli cells, and anti-Ki67 primary antibody (1:100; Abcam, Cambridge, UK) was used as a marker for cell proliferation. The following morning, sections were washed with PBS and then incubated with the secondary antibody provided in the VectaStain™ ABC kit (Vector Laboratories, Burlingame, CA, USA). After staining, the signals were visualized with DAB (Novus Biologicals, Littleton, CO, USA). All tissue sections were counterstained with haematoxylin for 30 seconds and washed in running water for 10 minutes. The slides were then dehydrated using a graded ethanol series and cleared in xylene. Finally, the slides were mounted, dried, and images were captured by microscopy. ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used for data analysis.

Cell viability

Cell viability was assessed by trypan blue staining (Thermo Fisher Scientific, Waltham, USA). Bovine ITT fragments were digested into a single cell suspension using a two-step enzymatic digestion as described previously [41]. In brief, ITT fragments were mechanically dissected using a disposable scalpel and needles in first enzymatic solution [0.1% w/v collagenase I-A (Sigma-Aldrich, St. Louis, USA) and 1% (vol/vol) Pen–Strep in Minimum Essential Medium (MEM)- α (Thermo Fisher Scientific, Waltham, USA)] followed by incubation at 37°C with gentle shaking. After a 10-minute settling period, the supernatant was collected, washed, and the sediment was subjected to further digestion in a second enzymatic solution [0.1% w/v collagenase I-A, 0.05% w/v DNAse I (Sigma-Aldrich, St. Louis, USA), 0.05% w/v hyaluronidase (Sigma-Aldrich, St. Louis, USA) and 1% (vol/vol) Pen–Strep in MEM-α], and incubated at 37°C with gentle shaking. The resulting dissociated cells were washed and suspended in fresh MEM-α with 1% Pen–Strep. The cell suspension was mixed with an equal volume of trypan blue staining solution and incubated for 2 minutes. Viable and non-viable cells were counted separately using a haemocytometer (Marienfeld Superior, Lauda-Königshofen Germany).

Total RNA extraction

Total RNA was extracted from frozen/thawed bovine ITT using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, USA) in accordance with the manufacturer's protocols. RNA was measured using a Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, USA) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, USA). RNA integrity was assessed by gel electrophoresis on a 1% agarose gel. Total RNA samples were stored at -80°C to await further analysis.

RT-qPCR

Table 1 lists the primers used in our study to investigate germ cell development in neonatal bovine testes. The target genes were *GFRα-1, PLZF, UCHL-1, C-KIT* and *THY1* for germ cells; *OCT4, NANOG* and *SOX2* for pluripotency/stem cells; *STRA8* and *CREM* for spermatogenesis; and *HSP70-2* for apoptosis. HSP70-2, a member of the Hsp70 family, exhibits selective expression in spermatogenic cells, and is known to play roles in germ cell apoptosis, male fertility and developmental regulation [8; 13; 14].

The SuperScript IV One-Step RT-PCR Kit (Thermo Fisher Scientific, Waltham, USA) was utilized for complementary DNA (cDNA) synthesis. Each reaction was performed in a total volume of 50 µl, comprising 25 µl of 2X Platinum™ SuperFi™ RT-PCR Master Mix, 100 ng of template RNA, 0.5 µl of each 50 µM forward and reverse primer, 0.5 µl of SuperScript™ IV RT Mix, and then brought to a final volume of 50 µl using nuclease-free water. This step was performed with a thermal cycler program, as follows: reverse transcription at 50°C for 10 minutes, denaturation at 98°C for 2 minutes, followed by 40 cycles of amplification at 98°C for 10 seconds, primer annealing for 10 seconds at 64.3°C, followed by 72°C for 30 seconds per kilobase (kb), and a final extension process at 72°C for 5 minutes.

A QuantStudio 3 system (Applied Biosystems, Foster City CA, USA) was utilized for all RT-qPCR reactions using 96-well plates. Each reaction consisted of a total volume of 20 µl per well, containing 4 µl of diluted cDNA templates, 10 µl of Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, USA), 1 µl each of 10 µM forward and reverse primers, and 4 µl of DNase/RNase free water. The RT-qPCR cycle program began with a pre-denaturation holding stage at 95°C for 20 seconds, followed by 40 cycles of amplification featuring denaturation at 95°C for 3 seconds, annealing/extension at 60°C for 30 seconds, and ended with a melt-curve stage at 95°C for 15 seconds and 60°C for 60 seconds. Samples were

run in triplicate along with a non-template control. *β-actin*, which has been identified and validated as a stable gene for RT-qPCR in bovine testes [26], was used as a housekeeping gene. Data were analysed using Excel (Microsoft, Redmond, WA, USA) and the $2^{-\Delta\Delta Ct}$ method [27].

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the quantitative data. If the data met the criteria for the normal distribution, statistical analysis was conducted using analysis of variance (ANOVA) followed by post hoc analysis employing Tukey's multiple comparisons test for group-wise comparisons. In cases where the data did not conform to a normal distribution, the Kruskal-Wallis test was utilized, followed by post hoc analysis employing Dunn's multiple comparisons test to identify differences between groups. All statistical analyses were conducted with GraphPad Prism version 9.3.0 for Mac software (GraphPad Software, San Diego, California, USA); $p < 0.05$ was considered statistically significant. Normally distributed data are presented as means ± standard deviations (SDs) while non-normally distributed data are presented as medians with 25% and 75% percentiles.

Results

The effects of transportation time on cell viability and tissue morphology

Tubular morphology was evaluated by H&E staining. Histological assessment revealed that the neonatal bovine testes contained undeveloped seminiferous tubules without a lumen (**Figure 2B**). The seminiferous cords in the two-week-old bovine testes mainly featured two primary cell types: germ cells and immature Sertoli cells. The germ cells and immature Sertoli cells were distinguished by their morphology, with germ cells characterized by their larger size,

rounder shape, pale cytoplasm, higher nuclear/cytoplasmic ratio, and the presence of one or two prominent nucleoli in the nucleus. In 2-week-old neonatal bovine testes, two types of germ cells were present in the seminiferous cords: gonocytes, located in the centre of the seminiferous cords and represented the majority of germ cells shortly after birth; and SSCs, which were found in the seminiferous cords adjacent to the basal lamina. The seminiferous epithelium was mostly occupied by immature Sertoli cells, which had basophilic nuclei. In addition, flat peritubular myoid cells (PMCs) were present in the interstitium surrounding the seminiferous cords, along with Leydig cells.

The proportion (%) of viable testicular cells derived from freeze/thawed testicular tissues was as follows: 83.10% ± 2.05% in the 1-hour group, 82.80% ± 4.88% in the 6-hour group, 78.59% ± 6.15% in the 24-hour group, and 72.40% ± 3.24% in the 48-hour group. There was no significant difference between the four groups with respect to cell viability (**Figure 2C**).

Analysis indicated a significant difference in the distribution of Grade 1, 2, and 3 tubules between the four groups ($p < 0.05$; **Figure 2D**. Testicular tissues processed after 48 hours featured a lower proportion of intact Grade 1 tubules when compared to the 1-hour group (29.53 ± 3.66% versus 65.61 ± 6.26%; $p = 0.0042$). The 48-hour group also featured a significantly higher proportion of Grade 2 cords with partial detachment from the basement membrane (54.04 ± 3.51% versus 27.21 ± 4.54% in the 1-hour group; $p = 0.0068$). Furthermore, the proportion of Grade 3 cords was 7.18 ± 1.94% in the 1-hour group, 14.10 ± 3.47% in the 6-hour group, 15.87 ± 1.87% in the 24-hour group, and 16.43 ± 2.14% in the 48-hour group. The seminiferous tubules in tissues from the 1-hour, 6-hour, and 24-hour groups appeared healthy in terms of their shape, size, colour, and integrity from H&E staining (**Figure 2E**). However, within the context of the 48-hour group, a notable alteration in the morphology of the seminiferous tubules was observed such as shrunken seminiferous cords. In addition, partial

rupture was evident in a proportion of these seminiferous tubules; this partial rupture could signify disruption in the structural cohesion of the tubules.

The effects of transportation time on SSCs

PGP9.5 immunohistochemical labelling detected germ cells in the seminiferous tubules of 2-week-old bovine testes. To investigate the effect of delayed processing time on germ cells, we applied immunohistochemical staining with the germ cell marker PGP9.5 to tissues processed at different time intervals (**Figure 3A**). PGP9.5-positive cells were mainly detected in the middle of the tubules (gonocytes); only a small proportion had migrated to the basement membrane (SSCs). In the 1-hour and 6-hour groups, PGP9.5-positive cells were round and well connected to or even touching PGP9.5-negative cells. However, in the 24- and 48-hour group, PGP9.5-positive cells had an irregular shape, and gaps between the PGP9.5-positive cells and the surrounding PGP9.5-negative cells were observed. The 24-hour group was less severely affected. The proportion of tubules containing PGP9.5-positive cells in the testicular tissue sections differed significantly among the four groups: 53.30 ± 10.38% in the 1-hour group, 50.17 ± 6.18% in the 6-hour group, 43.05 ± 6.96% in the 24-hour group, and 43.19 ± 6.45% in the 48-hour group (**Figure 3B**). Post hoc tests revealed a significant difference in the proportion of tubules containing PGP9.5-positive cells between the 6-hour and 48-hour groups ($p = 0.032$). The number of PGP9.5-positive cells per 10^4 μm^2 of seminiferous tubules showed a median of 5.3 and 25% – 75% quantiles [5, 10] cells in the 1-hour group, 5.0 [4.0, 10.0] in the 6-hour group, 5.0 [3.3, 6.7] in the 24-hour group, and 10 [6.7, 12.9] in the 48-hour group (Figure 3C). The number of PGP9.5-positive cells per unit of tubular area was significantly higher in the 48-hour group than in the other three groups ($p < 0.05$).

The effects of transportation time on Sertoli cells

Vimentin, a type of intermediate filament, is known to be expressed at low levels and in a narrow perinuclear region in postnatal bovine young-Sertoli cells [47]. Vimentin-positive areas in the testes included Sertoli cells and a few interstitial cells (**Figure 4A**). In 1-hour group and 6-hour group, the perinuclear region of the cytoplasm of Sertoli cells in seminiferous tubules showed positive staining for vimentin, with higher intensities observed on the basement membrane side than the centre of the cords. Gonocytes/SSCs (vimentin-negative), which have a higher nuclear/cytoplasmic ratio, exhibited close contact with vimentin-positive cells. In the 24-hour and 48-hour groups, there was an absence of vimentin in a narrow basal zone of Sertoli cells; this was not the case for the 1-hour and 6-hour groups. Instead, Sertoli cell nuclei were surrounded by a vimentin-positive zone. Vimentin-positive cells appeared shrunken and gaps were present between these cells and other vimentin-negative cells. Within the cord, the cells were no longer associated with other cells. In the 48-hour group, the boundaries of the seminiferous cords were difficult to recognize. The proportion of vimentin-positive cells in the seminiferous tubules was evaluated and no significant difference was found between the four groups (**Figure 4B**).

The effects of transportation time on cell proliferation

Ki67 was used to label proliferating cells in neonatal testicular tissue. Ki67-positive cells were mainly found within seminiferous tubules and some interstitial cells (**Figure 5A**). Most gonocytes located in the centre of seminiferous tubules were Ki67-negative. Ki67-positive cells within the seminiferous tubules were mainly located on the basement membrane. No significant differences in the proportion of Ki67-positive cells per tubule were detected in tissues processed after different delays (**Figure 5B**).

The effects of transportation time on gene expression

Finally, we performed RT-qPCR analysis on bovine ITTs to evaluate the effect of transportation time on the expression of genes related to gonocytes/SSCs, spermatogenesis and apoptosis. **Figure 6** shows changes in the expression of target genes in bovine ITTs in relation to transportation time. The expression levels of most genes did not show statistically significant differences when compared between groups, except for *C-KIT*. Analysis showed that *C-KIT* gene expression was significantly lower in the 6-hour, 24-hour and 48-hour groups than in the 1-hour group ($p < 0.05$).

Discussion

In this study, we followed clinical procedures for the cryopreservation of human ITT that are used routinely in the ORCTB at Oxford, UK, including transportation medium, storage conditions, cryoprotectant and the computational testicular cryopreservation programme used to freeze testicular tissue. Immediately upon acquisition, bovine ITT was sectioned into small fragments before immersion in transport medium; these fragments were similar in size to biopsies of human tissue collected from patients in clinic at Oxford. A previous study showed that the optimal preservation of testicular tissue morphology was achieved with a volume of tissue fragments around approximately 50 mm^3 or 80 mm^3, compared to ~6 mm^3 or 15 mm^3 [16]; however, this particular study did not consider tissue volumes between 15 mm^3 to 50 mm^3. Our study has contributed additional data on the cryopreservation of tissue fragments with a volume of approximately 30 mm^3. Our data showed that ITT can be maintained for 24 hours in HBSS-based tissue transport medium prior to cryopreservation without compromising viability, morphology, integrity, or the expression levels of key genes. However, we found that

391 a delayed processing time of up to 48 hours could lead to changes in tissue morphology,
392 including detachment of the seminiferous cords from basement membrane.

393 Cell viability is regularly used as an important evaluation parameter and a reliable predictor for
394 the health and potential development of tissues after cryopreservation [1]. The results of our
395 current study revealed that cell viability remained high after storage at 4°C for up to 48 hours,
396 with no statistically significant differences compared with tissues processed within 1 hour; this
397 indicated that ITTs could be transported in specific conditions for up to 48 hours at low
398 temperature before undergoing cryopreservation procedures without significant levels of cell
399 death. These results are consistent with a previous study on fragments of porcine testicular
400 tissue, which showed that the viability of the testicular cells and germ cells in fresh tissues
401 remained high when stored at 4°C for 48 hours, but was significantly reduced after storage for
402 72 hours [50]. Another study, involving human adult testicular tissues found that cell viability
403 remained high even after storage for 8 days in storage medium at 4°C [16]. Cell viability can
404 also be maintained at up to 80% when preserved in a cell suspension after being stored at 4°C
405 for 3 days [48; 49]. Previous research showed that the viability of testicular cells from 1-week-
406 old piglets varied significantly when preserved in different holding media, and found that
407 Leibovitz L15 (L15) with 20% FBS and HypoThermosol solution-FRS are two top preforming
408 medium [49]. Faes, K. et al. [15] demonstrated that, for adult human testicular tissue, viability
409 and cell apoptosis did not exhibit significant differences between the fresh control group and
410 any of the experimental medium conditions prior to cryopreservation. These experimental
411 medium included DMEM/F12, DMEM/F12 supplemented with 20% HSA, DMEM/F12
412 supplemented with 50% HSA, and the pure HSA medium. However, there was a progressive
413 deterioration observed in the structural integrity, morphology of Sertoli cells, and the average
414 number of spermatogonia per mm^2 as the HSA concentration increased. Given the variability
415 in the effects of different transport media, further investigation is warranted to ascertain the

optimal transport medium and temperature conditions for clinical applications involving human ITTs. The temperature at which tissues were stored before cryopreservation was essential and was reported to exert influence on the viability of cells; for example a study using mouse ITT showed that viability remained high after 24 hours at 4°C, but decreased significantly if stored at 22-24°C for 24 hours or 34°C for 6 hours [38]. In addition, another study showed that there were no significant differences in terms of viability, tubular morphology, and the morphology of Sertoli cells when comparing day 0 to any of the assessed temperatures (4°C, room temperature, and 37°C) after a storage period of 3 days [16]. However, a significantly higher incidence of cell apoptosis was detected in testicular tissue that had been stored for 3 days at 37°C when compared to the baseline (D0). The metabolic rate reduced by 50% for every 10°C reduction in temperature, with 10-12% of the original metabolic activity remaining at 4°C [40]. Therefore, it was suggested that storage at 4°C, a hypothermic temperature, can preserve biological tissue samples for short periods because the low temperature suppresses metabolism and reduces the activity of catabolic enzymes; as such, 4°C is often recommended for the transportation of tissues.

In both humans and the bovine model, the unique complex structure of SSC niches, in which the lumen has yet to develop in the seminiferous cords, provides adequate support for the maintenance and proliferation of SSCs. Therefore, preserving the intact structure of the SSC niche in seminiferous cords is important. In the present study, we compared the morphology of tissues stored for different transportation times. The results of H&E staining showed that the gap between the cords and the basement membrane increased after 48 hours of storage in transport medium at 4°C prior to cryopreservation. We also observed disordered tubular structure and rupture of seminiferous cords in tissues from the 48-hour group. These observations indicated that tissues structures could be preserved well and remained healthy over a 24-hour transport period, with high viability and good seminiferous cord structure,

whereas a transport period of 48 hours was more likely to result in abnormal morphology. Rupture of seminiferous cords and the increasing gap between seminiferous cords and the basement membrane could disrupt the SSC niche, affect cell-to-cell interaction, and reduce support for developing SSCs. This is first study to report that delayed processing could affect the morphology of frozen-thawed ITTs. Salian et al. provided evidence that 24 hours of storage at 4°C did not affect the morphology of testicular tissue in mice [38]. In a previous study, fresh adult human testicular tissues were shown to maintain high cell viability, a good tubular structure, and appropriate numbers of SSCs for 3 days at 4°C prior to cryopreservation [16]. However, significant deterioration of seminiferous tubular morphology was identified in fresh adult testicular tissues day 5 of storage, with disruption of the tubular structure, ruptured basement membranes, and the loss of the germinal epithelium. It is likely that ITT is more sensitive to changes in the environment than adult tissue, which demonstrated structural changes at an earlier timepoint. However, Faes et al.[15] used a scoring system that consisted of the average of several parameters without specifying when the early signs of tissue deterioration were apparent or which signs were specifically observed. The health classification criteria of testicular tissues vary between different studies; in the current study, the attachment of seminiferous cords and the basement membrane was evaluated and categorized according to strict criteria. The earliest changes in morphology were the presence of increasing gaps between the tubules and the basement membranes; this change was only slight in the 6-hour group but increased significantly after 48 hours of storage. As these gaps increase, the structure of the seminiferous cords is more likely to change, and the cells are more likely to become disordered and shrunken. Therefore, to better preserve the structure of the seminiferous cords, it is recommended that ITT is transported and cryopreserved within 24 hours of tissue collection.

For fertility preservation, our main goal is to preserve the SSCs as these must be maintained in order to restore fertility. In the present study, we evaluated SSCs in ITT by immunohistochemical staining with a PGP9.5 antibody and by analysing the expression levels of key genes, including *STRA8, PLZF, C-KIT, GFRA-1, THY1, UCHL-1, NANOG, OCT-4,* and *CREM.* The transcription and expression of *STRA8* is induced by retinoic acid (RA) and is considered a meiotic gatekeeper gene because it helps to regulate the initiation of meiosis during spermatogenesis [5; 24]. *PLZF* and *UCHL-1* are known to be expressed in all SSCs and therefore represent key markers [7; 9; 23; 45]. *GFRα-1* is expressed in more advanced SSCs and is associated with self-renewal and regulation owing to its important roles in activation of the GDNF-mediated receptor tyrosine kinase, the rearranged during transfection (RET) signalling pathway [31], and the neonatal gonocyte mitogen-activated protein kinase (MAPK)/extracellular receptor kinase (ERK) (MAPK/ERK) pathway [35]. *OCT-4, SOX2* and *NANOG* are markers of pluripotency, whilst Vimentin, which is expressed in the perinuclear area of Sertoli, peritubular, and a few interstitial cells, is proposed as a marker of Sertoli cells in bovine testis [12]. *THY1* is a surface marker for undifferentiated spermatogonial cells in bulls [37]. Our analysis showed that a 6 or 24-hour delay in processing had no significant effect on the numbers of SSCs or the expression levels of key SSC genes. However, the proportion of tubules containing SSCs decreased after 48 hours of storage in transport medium at 4°C, whereas the number of SSCs per area of seminiferous tubule increased. This difference was caused by changes in the structure of tubules after extended exposure to transport medium, including tubular rupture and shrinkage. Healthy and normal SSCs are characterized by their large round shape with a high nuclear/cytoplasmic ratio. However, changes in the shape of SSCs were observed in frozen-thawed tissues with a delayed processing time of 48 hours; this indicated that the morphology of SSCs was affected by extended transportation times. It is highly promising that the viability and structure of the SSCs was maintained effectively after

storage for 24 hours. A previous study, using porcine testicular tissues showed that the proportion of SSCs compared to whole testicular cells did not change significantly after 3 days of storage [49]. In another study, Zeng et al. assessed the functionality of SSCs from porcine testicular tissues by xenografting [50]; the survival of testis tissue grafted after a 48-hour cooling period remained high, and complete spermatogenesis and mature spermatozoa were detected. Overall, the condition of SSCs were maintained effectively for 24 hours prior to cryopreservation and no significant detrimental effects were detected. Further investigations of SSC functionality will require grafting or *in vitro* culture in the future.

In the present study, we evaluated the proliferation of Sertoli cells by immunohistochemistry using vimentin and Ki67 labelling. Ki67 has been used previously to label proliferating SSCs in ITT [34]. We found that most of the proliferating cells within the seminiferous cords of 2-week-old bovine ITTs were immature Sertoli cells. In humans, gonocytes migrate to the basement during the first few months after birth [10; 18], where they gain the ability to self-renew; during the transformation period, most gonocytes were Ki67-negative. The proportion of Sertoli cells and proliferating cells in the seminiferous cords did not change significantly over a 48-hour storage period. Furthermore, the morphology of Sertoli cells did not differ significantly. These results were consistent with previous findings showing that the morphology of Sertoli cells in human adult testicular tissues started to deteriorate from day 8 [15]. It is believed that a delay of 48 hours after tissue collection would not induce adverse effects in terms of the morphology of Sertoli cells and cell proliferation.

For gene expression, the expression of *CREM* in testicular tissues is known to be related to the potential ability of SSCs to undergo spermatogenesis and that the inactivation of *CREM* can result in the upregulation of downstream signalling molecules, causing post-meiotic arrest and promoting apoptosis [4; 28; 30]. *HSP70-2* is a molecular chaperone expressed in

514 spermatogenetic cells which maintains protein conformation and promotes the correct folding
515 and assembly of proteins into complexes [19]. *HSP70-2* knockout can lead to a reduction in
516 the number of spermatids owing to spermatogenic cell cycle arrest at the G2–M phase transition
517 and the induction of apoptosis [14]. *HSP70-2* has been shown to protect cells from apoptosis,
518 heat shock, and oxidative stress [3]. The results of the current study showed that the expression
519 levels of the spermatogenesis-related genes *CREM* and *HSP70-2* remained similar after storage
520 for 48 hours. The expression of *C-kit* during foetal gonadal development is associated with the
521 survival, migration, and proliferation of PGCs [21]. A previous study showed that *C-kit*
522 regulates the onset of the differentiation process of germ cells and is also a marker for
523 differentiating spermatogonia [51]. Testes undergo complex changes during the embryonic and
524 postnatal stages. In 2-week-old neonatal bovine testes, gonocytes migrate to undifferentiated
525 SSCs; thus, two types of germ cells are present. Some spermatogonia already exist in the
526 seminiferous cord; these can self-renew and are prepared for differentiation. The reduction in
527 *C-kit* gene expression observed in the current study may be associated with the suppressed
528 activity or loss of SSCs during the 48 hours of storage at low temperature, which might be
529 associated with increased apoptosis and subfertility [18]. Overall, our data suggest that a 48-
530 hour transport process would not adversely affect the spermatogenic potential of SSCs in
531 neonatal testes.

532 There are some limitations to our study that need to be considered. While bovine ITTs are a
533 valuable tool for investigating ITTs, they may not fully represent the complex biological
534 processes that occur in prepubertal human tissues. The protocols employed in the current
535 investigation, including the selection of cryoprotective medium, transport conditions, and
536 freezing procedure, closely mirrored the methodology utilized for preserving prepubertal
537 human tissue, but these may not be ideally suited for bovine tissue. Therefore, the findings
538 derived from this study may not fully represent the conditions and outcomes expected in human

tissue under these specific cryopreservation circumstances. In addition, we did not investigate the functionality of SSCs in the ITTs by xenotransplantation or *in vitro* spermatogenesis; these techniques could provide more comprehensive insight into the potential of SSCs for fertility restoration applications. Therefore, forthcoming investigations should aspire to replicate our outcomes utilizing prepubertal human tissues. Moreover, these studies should encompass additional experiments to fully elucidate the ramifications of distinct transport durations (up to 48 hours), transport conditions, size of tissue fragments, processing techniques, and cryopreservation methods, on the functionality of germ cells and other somatic cells within human ITTs.

Conclusions

In conclusion, our analyses demonstrated that a delay of up to 48 hours prior to cryopreservation does not have a significant impact on cell viability and the expression of key genes related to SSCs and apoptosis in frozen-thawed bovine ITT. This finding is important because it suggests that researchers and clinicians can potentially extend the window for ITT processing and storage up to 48 hours; this could improve accessibility to the limited number of specialist units providing testicular tissue cryopreservation for fertility preservation. However, our study also revealed that delayed processing is associated with morphological changes, specifically the detachment of seminiferous cords from the basement membrane. Therefore, caution should be exercised when considering delayed processing of ITT. Further studies are required to investigate the underlying mechanisms and potential consequences of these morphological changes. Overall, our findings provide valuable insights into the optimal handling and processing of ITT for research and clinical applications.

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Declaration of competing interest

None of the authors have any conflicts of interest to declare.

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Figure legends

Figure 1. Study design and tissue preparation. (A) Bovine prepubertal testicular tissues were dissected into small fragments and held in transport medium for 1 hour (no delay), 6 hours, 24 hours, or 48 hours. Tissue fragments were cryopreserved using the standard testicular tissue cryopreservation procedure at the Oxford Reproductive Tissue Cryopreservation Programme (ORTCP) and then stored in liquid nitrogen. After thawing, we analyzed testicular cell viability and apoptosis, and performed histological and immunohistochemical analyses. We also used real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) to investigate the expression of key genes. (B) Images of testicular tissue showing the tunica vaginalis and epididymis (left), after removal of the tunica vaginalis and epididymis (middle), and after a longitudinal incision to expose the testicular parenchyma (right). Arrow: tissue

763 fragments of 3 × 3 × 3 mm^3 in size were taken from the region indicated by the white arrow
764 for subsequent cryopreservation. H&E = hematoxylin and eosin; IHC =
765 Immunohistochemistry; RT-qPCR = real-time quantitative reverse transcription-polymerase
766 chain reaction.

767

768 Figure 2. Effect of delayed processing on cell viability and morphological grading of immature
769 testicular tissue. (A) Three categories were assigned based on the gap between the seminiferous
770 tubules and the basement membrane. Scale bar = 50 μm. (B) Representative histology image
771 of fresh bovine neonatal testicular tissues stained by hematoxylin and eosin (H&E). SSC =
772 Spermatogonial stem cells; PMC = peritubular myoid cell. Scale bars = 20 μm. (C) Cell
773 viability in freeze/thawed neonatal testicular tissues processed after 1 hour, 6 hours, 24 hours,
774 and 48 hours. (D) Proportion of seminiferous cords categorized as Grade 1, 2, or 3. Significant
775 differences between two groups, as determined by post hoc tests: ** $p<0.01$; *** $p<0.001$. (E)
776 Representative images of H&E staining of tissues processed after 1 hour, 6 hours, 24 hours,
777 and 48 hours. Scale bars = 50 μm.

778

779 Figure 3. Effect of delayed processing on germ cells in cryopreserved-thawed immature
780 testicular tissue. (A) Representative images of immunohistochemical staining for PGP9.5
781 showing gonocytes inside bovine neonatal seminiferous tubules in samples processed after 1
782 hour, 6 hours, 24 hours, and 48 hours. Scale bar = 50 μm. (B) Proportion of tubules with
783 PGP9.5-positive cells per tissue section. The data were normally distributed, and one-way
784 ANOVA was performed. (C) The number of PGP9.5-positive cells per 10^4 μm^2 within
785 seminiferous tubules. The data are presented as median, min to max. The data were not

normally distributed and therefore the Kruskal-Wallis test was performed. For B and C, overall significance between the four groups is denoted by # $p<0.05$. Significant differences between the two groups are shown, as determined by post-hoc tests: *$p<0.05$; ** $p<0.01$; *** $p<0.001$; ****$p<0.0001$.

Figure 4. Effect of delayed processing on Sertoli cells in cryopreserved-thawed immature testicular tissue. (A) Representative images showing the immunohistochemical staining of vimentin. Images show Sertoli cells inside bovine neonatal seminiferous tubules in cryopreserved-thawed samples processed after 1 hour, 6 hours, 24 hours, and 48 hours. Scale bar = 50 µm. (B) Proportion of vimentin-positive cells in seminiferous tubules. The data are presented as the mean ± SD and significance was determined by one-way ANOVA. *$p<0.05$; ** $p<0.01$; *** $p<0.001$; ****$p<0.0001$.

Figure 5. Effect of delayed processing on cell proliferation in cryopreserved-thawed immature testicular tissue. (A) Representative images showing the immunohistochemical staining of Ki67-positive (proliferating) cells in testicular tissue processed after 1 hour, 6 hours, 24 hours, and 48 hours. Scale bar = 50 µm. (B) The quantification of Ki67-positive cells per seminiferous tubule. The data are presented as the mean ± SD and one-way ANOVA was performed to determine the significance of differences. *$p<0.05$; ** $p<0.01$; *** $p<0.001$; ****$p<0.0001$.

Figure 6. Effect of delayed processing on the expression of key genes in cryopreserved-thawed immature testicular tissue as determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The effects of various processing delays on the

809 expression of *STRA8, PLZF, C-KIT, GFRA-1, THY1, UCHL-1, NANOG, OCT-4, CREM, and*
810 *HSP70-2* in freeze/thawed bovine ITT. Error bars represent the standard deviation among
811 biological replicates (N=3). * $p<0.05$ in post-hoc test from one-way analysis of variance
812 (ANOVA).

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