RESEARCH ADVANCES IN MALE FERTILITY: NEW HORIZONS FOR INVESTIGATING HUMAN TESTICULAR FUNCTION AND DEVELOPMENT OF CLINICAL FERTILITY PRESERVATION APPROACHES

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RESEARCH ADVANCES IN MALE FERTILITY: NEW HORIZONS FOR INVESTIGATING HUMAN TESTICULAR FUNCTION AND DEVELOPMENT OF CLINICAL FERTILITY PRESERVATION APPROACHES

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Editorial: Research advances in male fertility: New horizons for investigating human testicular function and development of clinical fertility preservation approaches

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Editorial on the Research Topic:

Research advances in male fertility: New horizons for investigating human testicular function and development of clinical fertility preservation approaches

The topic for this special issue on Research Advance in Male Fertility was defined and initiated by the three authors in 2021 inviting submissions to address a broad range of original research papers and reviews. Ten submissions involving 64 authors were published after peer review between October 2021 and July 2022. The accepted two reviews and eight original papers present a wide array of themes and topics describing new and exciting strategies from basic science to clinical applications (Figure 1). As of mid-August 2022, the submissions attracted already 12.000 views and 4.000 downloads of the open access submissions.

The early response reveals that male fertility preservation is currently a hot topic in the field of reproductive medicine. This is because poor male reproductive health leads to nearly half of failed pregnancy attempts. Declining semen quality over the past 50 years indicates the role of changing lifestyle and exposure to environmental and toxic components as potential contributing factors. Sperm retrieval is feasible in adult infertile men by using surgical procedures like TESE (Testicular sperm extraction) and can be combined with cryopreserving semen samples for future Assisted Reproductive Technologies (ART) treatments. Prepubertal patients undergoing gonadotoxic treatment to cure malignant or non-malignant diseases or patients with specific genetic

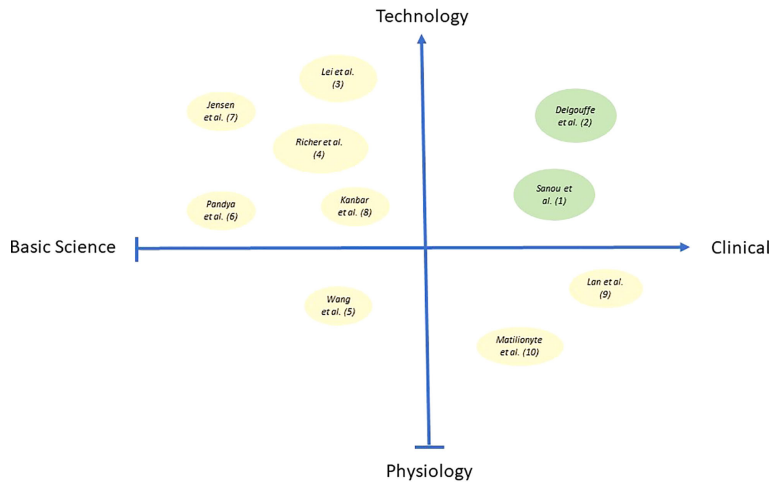

FIGURE 1
Bubble plot in the figure illustrates the publications from this issue encompassing the quad-spectrum of basic science research, clinical research, physiological and technology-based research. X-axis represents Basic science to Clinical research, and Y-axis represents Physiology to Technology-based research. Bubbles in green color represent the two reviews, and bubbles in pale-yellow color represent eight original manuscripts published as part of this special issue. Different bubble size indicates the impact of specific publications w.r.t citations.

abnormalities are at high risk of losing their fertility. Those patients have currently no option for fertility protection or means of fertility preservation to father offspring. Using established strategies, fertility and andrology centers began to cryo-bank immature testis tissue from biopsies to offer fertility preservation strategies that are based on preserving spermatogonial stem cells (SSCs) rather than spermatozoa. While cryobanking occurs, strategies for the generation of gametes from banked tissues do not yet exist for clinical applications but have been developed in animal studies.

This Research Topic focuses therefore on research advances in the field of fertility preservation and the development of strategies to investigate the impact of environmental factors on male reproductive health. A special focus is put also on the development of new experimental strategies to generate male gametes *ex vivo*. The rapidly evolving field is reviewed, and two-dimensional and three-dimensional culture conditions, including organoid and organ culture models, are presented for several species including monkeys.

We have observed a mix of submissions covering all aspects from basic science to clinical applications. The submissions cover themes describing novel insights into physiology but also new technical advances and exciting technology. The figure above depicts the range of contents as a plot showing the two reviews (green dots) and eight original research papers (grey dots) in relation to their content. The size of dots relates to the number of citations in each submission indicating the depth of scientific information in each report (Figure 1).

Both reviews reveal excellent overviews of the scientific state of the art. Sanou et al. from the Amsterdam University Medical Centre provide an insight into the status of basic research in the area of SSC-based therapies and create an outlook into innovative clinical strategies which can be generated in the future. Although still experimental, biobanking of immature testicular tissue has already become nearly a routine strategy for males facing high gonadotoxic treatments prior to puberty. It is envisaged that the spermatogonia in these cryopreserved samples have the potential of forming spermatids *via* various means and thereby may be used for therapeutic applications later in life. The authors reviewed the past 30 years of research in this field and describe crucial milestones like the transplantation of spermatogonia, grafting of testicular tissue, and different *in vitro* and *ex vivo* culture settings. They claim that it is now time to take the next step in initiating the first clinical applications and trials. Taking into consideration the effectiveness of the SSC-based therapies and addressing hurdles and risks they see safe and effective SSC-based fertility treatments to be implemented in the coming years.

A more clinical perspective on this topic is provided by Delgouffe et al. from the Vrije Universiteit Brussel. Their review addresses the relevant question of inclusion and exclusion criteria for patients when novel treatment options for stem cell-based fertility preservation in boys are established. Today, an increasing number of young patients are at risk of germ cell loss and many centers have started to cryopreserve immature testis tissue. While the methodology was originally considered to

be predominantly offered to young cancer patients prior to gonadotoxic chemo- or radiotherapy, it has been realized that banking of testicular tissue is nowadays offered to many patients with non-malignant conditions. These patients arise in the context of conditioning therapy prior to hematopoietic stem cell transplantation (HSCT) and bone marrow transplantation (BMT). A considerable number of patients have genetic or developmental disorders leading to prepubertal germ cell loss and may also be considered for tissue banking. In addition, a significant group of patients is banking after they faced previous exposure to chemo- or radiotherapy. The authors address the question if all patients should be considered for surgical removal as this invasive and still experimental procedure has inherent risks. It could be argued that priority should be given to patients with a significant risk of becoming infertile. The authors review the existing evidence and propose that testicular tissue banking should be offered to young cancer patients in need of high-risk chemo- and/or radiotherapy irrespective of previous low-risk treatment and to patients with non-malignant disorders facing high-risk conditioning therapy. A small group of patients with bilateral cryptorchidism may also be recommended for retrieval and banking. Patients facing medium- to low-risk gonadotoxic exposures usually maintain their fertility and may not be considered for such strategies as are Klinefelter patients for whom the perspectives to undergo routine TESE procedures are more promising than cryobanking of extremely limited SSCs.

Eight original submissions provide significant insights and novel datasets on many significant aspects in the field of male fertility preservation. In the study of Lei et al., the authors considered problems with adequate meiosis in germ cells undergoing spermatogenesis *in vitro*. They express concerns that incomplete synapsis of the homologous chromosomes or impaired homologous recombination may lead to failure of crossover formation and subsequent chromosome nondisjunction. The risk for aneuploid sperm may rise in such *ex vivo* generated gametes. The authors describe the meiotic checkpoints usually eliminating aberrant spermatocytes during spermatogenesis, and present evidence that *in vitro* derived meiotic cells undergo meiosis despite incomplete chromosome synapsis and in the absence of XY-body or crossover formation. They propose to apply an improved *in vitro* system in which the transition of germ cells through various positions in the seminiferous epithelium is mimicked. Despite obvious improvement in meiotic progression, the authors describe that meiotic recombination was still disturbed with only a few meiotic crossovers. Furthermore, they conclude that meiotic checkpoints may not be fully functional under *in vitro* conditions and propose to closely monitor the meiotic process to avoid the generation of aneuploid gametes when such systems are considered for clinical applications.

Shortcomings associated with *in vitro* generation of germ cells are also addressed by Richer et al. Survival of germ cells is limited, and tissue degeneration is prominent in testicular organotypic cultures. The author performed experimental studies generating different 2D and 3D organotypic systems using two different mouse strains. The organoids contained tubule-like structures supporting long-term survival and differentiation of germ cells to the meiotic phase as well as functioning Leydig cell. 3D scaffolds enabled improved spheroidal morphology and generation of functional units revealing both, spermato- and steroidogenesis without the survival of germ cells in long-term culture. Overall, the study implies that further optimization of culture conditions is required. The authors assume that such systems may become valuable tools for studying the interplay of somatic and germ cells and may lead to the development of therapies for male infertility.

Another experimental strategy is described in the study of Wang et al., presenting data on germ cell homing and development following xenotransplantation of human germ cells into the mouse testis. Concerns that *in vitro* propagation of spermatogonia may cause genetic and epigenetic changes prompted the authors to change the strategy and inject germ cell suspensions into the testis without an initial expansion in culture. This strategy may be safer and easier for future clinical applications. Primary cell suspensions from 11 infant testes with cryptorchidism were labeled with green fluorescent dye and directly infused into seminiferous tubules of busulfan-treated mice. Six to nine weeks later whole mount analysis was performed to detect gonocytes and spermatogonia using immunofluorescence staining for MAGEA4, GAGE, UCHL1, SALL4, UTF1, and LIN28 and two somatic cell markers (SOX9 and CYP17A1 for Sertoli and Leydig cells, respectively). Homing of human gonocytes and spermatogonia in mouse seminiferous tubules was observed with a colonization efficiency of approx. 6%. The authors conclude that colonization of mouse seminiferous tubules by human germ cells can be achieved without prior *in vitro* propagation. This observation may simplify the necessary steps required for a successful germ cell transplantation and may reduce the risk of inducing mutations. However, the preliminary observations need to be confirmed and substantiated by additional studies showing that the low number of transplanted SSC is sufficient to secure the presence of sperm in the ejaculate of those patients over time.

Experimental studies exploring how hypothermia during tissue handling prior to organ culture affects the epigenetic integrity of cells in the immature testes were addressed in a study by Pandya et al. The impact of hypothermic holding was analyzed in tissue obtained from 6-day-old mice. As endpoints, relative mRNA expression of DNA methyltransferases Dnmt1, Dnmt3a, and Dnmt3b along with global DNA methylation were analyzed after systematic exposure to 4°C cold medium for 6 and 24 hours. Endpoints were analyzed in mouse testis tissue after

two weeks of organotypic culture. No significant variation in methyltransferase expression was detected in relation to the two time periods of exposure. Global DNA methylation was unchanged between 0, 6, and 24 h exposed tissues. Authors conclude that hypothermic holding of immature testis tissue for up to 24 hours does not affect DNA methylation providing experimental evidence that this procedure may also be safe in clinical settings.

Jensen et al. studied the diverse phenotype of testes from infertile men with non-obstructive azoospermia (NOA). These patients show impaired spermatogenesis in dilated and undilated atrophic seminiferous tubules. Active spermatogenesis occurs more frequently in dilated tubules. In this study, attention was focused on the un-dilated tubules and their SSC microenvironment. Analysis of 34 testis tissue samples obtained from undilated areas was performed. Initial analysis revealed hypo-spermatogenesis in five, maturation arrest in 14, and Sertoli cell only in 15 samples. Five control samples from fertile men were also analyzed. Methods to determine the endpoints were routine histology, RT-PCR, and immunofluorescence of germ and Sertoli cell markers. Irrespective of the severity of spermatogenic depletion un-dilated tubules showed an increase in Anti-Müllerian hormone mRNA and protein expression compared to the control. Other markers like GDNF and BMP4 showed variable expression depending on the histological findings at the mRNA and protein level. NOA testes revealed a reduction of germ cell markers DDX4 and MAGE-A4. Changes were also observed in the number of androgen receptor positive Sertoli cells and in mRNA expression. It was concluded that somatic and germ cells are differentially affected indicating different and individualized mechanisms leading to testicular dysfunction. This may require individualized regimens for different damage types when *in vitro* conditions for germ cell production will be defined in the future.

Experimental studies using exciting new microfluidic technology and piglets as model species were performed by Kanbar et al. During the last decades, the successful *in vitro* maturation of immature testicular tissue using organotypic tissue culture in mice has been reported. However, this approach remains challenging in larger mammals and primates. Here the research implemented advances in biomaterial technology creating complex culture systems to better mimic *in vivo* conditions. A comparison of four different organotypic tissue culture systems was performed with a maximal culture period of 30 days. Histological observations and detection of markers provided a large array of endpoints in the tissues. The piglet testis fragments developed all tested systems indicated by the growth of seminiferous tubules, expansion of Sertoli and germ cells, and release of testosterone. While some systems were more efficient, several physiological parameters showed identical progress in all used systems. The authors conclude that microfluidic systems need to be further optimized together with culture media to establish the most favorable conditions for *ex vivo* spermatogenesis in dissected immature testes.

As part of this special topical issue focusing on 'research advances in fertility preservation', two original papers were submitted reporting clinical observations. Lan et al. described the success rates when Intra-Cytoplasmic Sperm Injection (ICSI) - Invitro Fertilization (IVF) was performed on patients with non-obstructive azoospermia (NOA). Sperm retrieval rates and ICSI outcomes after micro-TESE in NOA patients remain inconclusive providing no solid base for conducting comprehensive recommendations. The paper reports retrospective data on 968 NOA patients undergoing micro-TESE. Embryological, clinical, and live birth outcomes were analyzed taking various clinical parameters into consideration. Overall sperm retrieval rate was 44.6% enabling to perform ICSI in 299 couples. As an outcome, 150 clinical pregnancies and 140 live-birth deliveries were achieved. Frozen and fresh sperm cycles showed similar outcomes. NOA patients with Azoospermia Factor c (AZFc) microdeletions showed the lowest rate of success. When fewer than 20 sperm were retrieved, the outcome was significantly reduced compared to cycles with more than 20 sperm available for ICSI.

A different clinical dataset was reported by Matilionyte et al., exploring the effects of chemotherapy exposure during pregnancy. The study aimed to describe platinum-based alkylating-like chemotherapeutic exposures on germ cell numbers in the human fetal testis. Specific focus was put on the effects at variable gestational ages of the fetus. The ability to explore such parameters in human fetal testis cultures as a model for chemotherapy exposure during pregnancy may open novel experimental strategies. A total of 23 human fetal testicular tissue samples were available for *in vitro* culture. Three different gestational age groups were defined. Cisplatin or vehicle exposure occurred in culture for 24 hours with analyzing the tissues 72 and 240 hours later. Endpoints analyzed were numbers of gonocytes and (pre) spermatogonia. Depending on gestational age, gonocyte numbers were reduced after 72 hours. At 240 hours post-exposure, gonocytes and spermatogonia showed a reduction in testicular tissues obtained from fetuses in the mid- and late-second trimester, whilst remaining unaffected in early-second trimester tissues. The authors conclude that *in vitro* culture of human fetal testicular tissues presents a promising model system to study the effects of chemotherapy exposure in fetal testes.

In summary, the collection of articles included in this Research Topic on "*Research Advances in Male Fertility: New Horizons for Investigating Human Testicular Function and Development of Clinical Fertility Preservation Approaches*" provides novel and important insights for all researchers and healthcare professionals interested in the field of male reproduction. It will hopefully inspire new investigations and exciting research questions in this important field of testicular research.

Author contributions

All authors contributed to the drafting, revision, proof-reading and final approval of the editorial.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Meiotic Chromosome Synapsis and XY-Body Formation *In Vitro*

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To achieve spermatogenesis *in vitro*, one of the most challenging processes to mimic is meiosis. Meiotic problems, like incomplete synapsis of the homologous chromosomes, or impaired homologous recombination, can cause failure of crossover formation and subsequent chromosome nondisjunction, eventually leading to aneuploid sperm. These meiotic events are therefore strictly monitored by meiotic checkpoints that initiate apoptosis of aberrant spermatocytes and lead to spermatogenic arrest. However, we recently found that, *in vitro* derived meiotic cells proceeded to the first meiotic division (MI) stage, despite displaying incomplete chromosome synapsis, no discernible XY-body and lack of crossover formation. We therefore optimized our *in vitro* culture system of meiosis from male germline stem cells (mGSCs) in order to achieve full chromosome synapsis, XY-body formation and meiotic crossovers. In comparison to previous culture system, the *in vitro*-generated spermatocytes were transferred after meiotic initiation to a second culture dish. This dish already contained a freshly plated monolayer of proliferatively inactivated immortalized Sertoli cells supporting undifferentiated mGSCs. In this way we aimed to simulate the multiple layers of germ cell types that support spermatogenesis *in vivo* in the testis. We found that in this optimized culture system, although independent of the undifferentiated mGSCs, meiotic chromosome synapsis was complete and XY body appeared normal. However, meiotic recombination still occurred insufficiently and only few meiotic crossovers were formed, leading to MI-spermatocytes displaying univalent chromosomes (paired sister chromatids). Therefore, considering that meiotic checkpoints are not necessarily fully functional *in vitro*, meiotic crossover formation should be closely monitored when mimicking gametogenesis *in vitro* to prevent generation of aneuploid gametes.

Keywords: *in vitro* spermatogenesis, *in vitro* meiosis, spermatogonial stem cells (SSCs), Sertoli cells, spermatocytes, fertility preservation

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INTRODUCTION

With the aim of potential future applications, such as male fertility preservation or treatment, many laboratories worldwide have attempted to generate functional sperm *in vitro*. However, *in vitro* spermatogenesis culture systems that fulfill the "gold standards" of *in vitro*- derived germ cells, for instance proper meiotic chromosome organization and recombination, and viable euploid offspring (1),

have barely been reported. One study, using mouse embryonic stem cells (ESCs) and neonatal testicular cells, was able to recapitulate most of these key events (2). Especially meiosis, the cell division in which DNA replication is followed by two successive rounds of chromosome segregation (MI and MII) to give rise to genetically diverse haploid gametes, seems to be particularly challenging to replicate *in vitro*. Meiotic problems, for instance impaired synapsis and recombination of the homologous chromosomes during the first meiotic prophase, are considered as the main factors causing chromosome nondisjunction and subsequent aneuploidy in sperm (3–5). Aneuploidy in human gametes can ultimately cause genomic instability, infertility, recurrent pregnancy loss and developmental defects such as Klinefelter's syndrome (6–8). Many studies have assessed aneuploidy in sperm of infertile and fertile men, and aneuploidy levels appear to be significantly higher in infertile men (9–11). To prevent chromosomal aberrations from being transmitted to the offspring, meiotic prophase checkpoints exist to timely eliminate aberrant spermatocytes before entering the meiotic M-phase stage (12).

In spermatocytes *in vivo*, the first meiotic prophase is tightly regulated and prolonged. Early during meiosis, about 200-400 programmed DNA double-strand breaks (DSBs), and subsequent meiotic recombination sites, are initiated, which are required for the initiation of homologous chromosome synapsis (13). Subsequently, while chromosome synapsis is being completed, these DSBs sites are repaired *via* meiotic recombination, ultimately leading to the formation of at least one or two meiotic crossovers per homologous chromosome pair (14). These meiotic crossovers not only exchange genetic material between non-sister chromatids, but also form physical links between homologous chromosomes, called chiasmata, that ensure proper chromosome segregation at M-phase I (15). To ensure proper crossover formation, homologous chromosome synapsis and recombination are strictly monitored by meiotic prophase checkpoints (12). Also in human spermatocytes, asynapsis of the homologous chromosomes or failure of proper meiotic DSB repair results in checkpoint activation and eventually meiotic prophase arrest (16). However, these meiotic checkpoint and arrest mechanisms appeared not to be fully functional during meiosis *in vitro* (17).

Several studies have described complete spermatogenesis *in vitro* using various cell culture strategies and starting cell types, such as pluripotent stem cells (PSCs) (2, 18–21), or spermatogonial stem cells (SSCs) (17, 22–24). Of these studies only few characterized whether key meiotic events took place *in vitro* (2, 17). When mouse embryonic stem cells (ESCs), grown on a cell suspension of male neonatal gonad, were induce to complete *in vitro* meiosis, no meiotic problems were reported (2). However, for human fertility treatment or preservation, patient-specific ESCs or neonatal gonadal cells are not available. Therefore, to circumvent the use of ESCs or neonatal gonad, we used mouse spermatogonia, maintained in culture as mouse male germline stem cells (mGSCs), which can be induced to undergo spermatogonial differentiation *in vitro* by using retinoic acid (RA) treatment (25). Moreover, when subsequently placed on a feeder layer of immortalized Sertoli cell line, these can complete meiosis *in vitro* (17). However, many key meiotic events, such as chromosome synapsis, XY-body formation and crossover formation, were not completed. Despite this, many of these *in vitro*-generated spermatocytes were not eliminated by meiotic prophase checkpoints that are active *in vivo*, and still proceeded to the meiotic metaphase stages (M-phase), occasionally forming spermatid-like cells (17). Due to the lack of meiotic crossovers, these M-phase spermatocytes displayed univalent chromosomes (pairs of sister chromatids), instead of bivalents (pairs of homologous chromosomes), which is a typical character of chromosome nondisjunction, and will most likely lead to aneuploid sperm (26).

In order to achieve *in vitro* meiosis with complete chromosome synapsis, XY-body formation and crossover formation, we adapted our previous culture system to more closely mimic the *in vivo* situation. *In vivo*, multiple germ layers, including undifferentiated SSCs, differentiating SSCs and spermatocytes, are spatio-temporally organized at the basal lamina of the seminiferous tubules in the testis, leading to the continuous production of spermatids. However, in our previous *in vitro* culture system, only one germ layer was present at a certain time. Meanwhile, we also observed that many dead SK49 Sertoli cells appeared 6 days after induction. Therefore, to mimic the normal *in vivo* parallel development of different developmental germ cell subtypes, we re-plated *in vitro*-generated spermatocytes to a fresh plate containing a new layer of proliferatively inactivated Sertoli cells supporting fresh undifferentiated mGSCs. We now observed that, even without addition of undifferentiated mGSCs, freshly plated Sertoli cells support complete meiotic chromosome synapsis and XY body formation during *in vitro* meiosis. However, although crossovers now could be detected, meiotic recombination was still sub-optimal *in vitro*, leading to very few crossovers in comparison to the *in vivo* situation.

MATERIALS AND METHODS

Animals

Neonatal (4-8 d.p.p) DBA/2J male mice were used for isolation of primary spermatogonia (male germline stem cells, mGSCs) as described previously (27, 28). All animal procedures were in accordance with and approved by the animal ethical committee of the Amsterdam UMC, Academic Medical Center, University of Amsterdam.

Male Germline Stem Cells and Sertoli Cell Line Culture

Mouse GSCs were cultured as previously reported (25, 27, 29). The cells were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs; Gibco, A34962), using a medium (medium I) composed of StemPro-34 SFM medium (Thermo Fisher Scientific), StemPro-34 Supplement (Thermo Fisher Scientific), 1% fetal bovine serum (FBS), recombinant human GDNF (10 ng/ml, 450-10, Peprotech), recombinant human bFGF (10 ng/ml, 100-18B, Peprotech), recombinant human EGF (20 ng/ml, AF-100-15, Peprotech), recombinant human LIF (10 ng/ml, CYT-644, Prospec), as well as other

components as previously reported (29) and described in **Supplementary Table 1**. The cells were refreshed every 2-3 days, and passaged every 5-7 days at a ratio of 1:4-6 on freshly plated mitotically inactivated mouse embryonic fibroblasts. The cells were maintained at 37°C in 5% CO2 in air.

As a feeder cell to support *in vitro* meiosis of mouse mGSCs, we used an available immortalized Sertoli cell lines SK49 (30). The cells were cultured at 37°C and 5% CO2 in Dulbecco's Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL).

In Vitro Meiosis of mGSCs

SK49 cells, inactivated by mitomycin (10μg/mL, M7949, Sigma), were grown on 12-well plates pre-coated with laminin (20 μg/mL, L2020, Sigma) to a density of 1 x10^5 per well. Then mGSCs were seeded on these Sertoli cells to maintain mGSCs proliferation for two days at 37°C using medium I, composed of as described previously (17, 29). To induce meiosis, the cells were cultured at 34°C. From day 0 to day 3, medium was changed to medium II, composed of StemPro-34 SFM medium and StemPro-34 Supplement, 10% KnockOut Serum Replacement (KSR), 5% fetal bovine serum (FBS), Retinoic acid (RA) (1μM, R2625, sigma), Recombinant Mouse BMP-4 Protein (20 ng/mL, 5020-BP, R&D Systems), Recombinant Mouse Activin A Protein (100 ng/mL, 338-AC, R&D Systems), and other components described in **Supplementary Table 1**. Starting from day 3 after meiosis induction, medium was changed to medium III, composed of StemPro-34 SFM medium and StemPro-34 Supplement, 10% KSR, 5%FBS, RA (1μM), Bovine Pituitary Extract (BPE) (30μg/mL, 13028014, Thermo Fisher Scientific), Follicle-stimulating hormone (FSH) (100 ng/mL, F4021, Sigma), Testosterone (5 μM, 86500, Sigma), and other components described in **Supplementary Table 1**, which was refreshed daily. From day 4 to day 6, the same amount of additional 12-well plates was prepared and pre-coated with laminin. Then undifferentiated mGSCs were grown on SK49 Sertoli cells in medium I. At day 6, the *in vitro*-induced meiotic cells were dissociated by accutase (Thermo Fisher Scientific), and were subsequently seeded on the additional 12-well plates containing undifferentiated mGSCs and SK49 cells. All cells were subsequently cultured in medium III. For the control group without undifferentiated mGSCs, the *in vitro*-induced germ cells grow on SK49 Sertoli cells using medium III. From day 6 to day 15, cells were collected for immunocytochemistry.

Immunocytochemistry

Meiotic spread preparations were prepared as previously described (31). Alternatively, the cells were spread on the slides using a Cytospin (CELLSPIN, 521-1990, VWR) (17). Briefly, *in vivo* spermatocytes were yielded from seminiferous tubules as previously described (31). Then these spermatocytes were washed three times with 1x phosphate buffered saline (PBS) and diluted in 200 μL PBS/1% BSA containing 30,000 to 50,000 cells for each cytospin spot and spun for 7 minutes at 112g. Similarly, *in vitro*-induced germ cells were detached from the culture dish using 0.25% trypsin, and transferred to microscope slides by Cytospin. The slides were air dried for 10min, fixed in 4% PFA and stored at 4°C in PBS or stored at -80°C after air drying.

Immunocytochemistry was performed as described previously (17). Omission of the primary antibodies and replacement with mouse, rabbit and sheep isotype IgGs were used as negative control. Primary antibodies and secondary antibodies are described in **Supplementary Table 2**.

Microscopy

Fluorescence microscopy images were acquired using a Plan Fluotar 100×/1.30 oil objective on a Leica DM5000B microscope equipped with a Leica DFC365 FX CCD camera. Images were analyzed using Leica Application Suite X and Image J version Java 1.8.0_77. The figures and graphs were constructed using Graphpad prism, Adobe Photoshop CS5 version 13.0.1 and Adobe illustrator version CS6.

Statistics

For imaging and quantification of meiotic cell types at all different time points, 3 microscope slides for each time point from three independent experiments were assessed. For quantification of RAD51, 13 testicular (*in vivo*) pachytene spermatocytes and 13 *in vitro*-derived pachytene spermatocytes were assessed. For MLH1, 12 testicular (*in vivo*) pachytene spermatocytes and 13 *in vitro*-derived pachytene spermatocytes were assessed. Statistical significances between the number of foci were determined by applying the Student's t-test.

RESULTS

Complete Meiotic Homologous Chromosome Synapsis *In Vitro*

To optimize chromosome synapsis, XY-body formation and meiotic recombination in our *in vitro* spermatogenesis system, we provided a "second wave of *in vitro* spermatogenesis" by re-plating *in vitro*-generated early meiotic cells to an undifferentiated layer of GSCs growing on freshly plated proliferatively inactivated SK49 Sertoli cells. The culture system now consists of three time periods with three different culture media that represent: (1) spermatogonial self-renewal, (2) spermatogonial differentiation and initiation of early meiosis, (3) co-culture of meiotic cells with GSCs (**Figure 1A**). Spermatogonial self-renewal, differentiation and initiation of meiosis were induced by using medium I and medium II, respectively, exactly as reported previously (17). Early meiosis was further supported by a third medium (medium III) that, besides follicle-stimulating hormone (FSH), testosterone, and bovine pituitary extract (BPE), also contained retinoic acid (RA). After an induction period of 6 days, the *in vitro*-generated spermatocytes were collected and seeded on plates containing GSCs and SK49 Sertoli cells that were pre-cultured for two days using medium I to maintain self-renewal. Medium III was subsequently used to support further meiotic progression of the already *in vitro*-generated spermatocytes and simultaneously initiate the differentiation of the fresh layer of GSCs. To assess meiotic progression, we collected the cell samples at days 6, 8, 10, 12, 14

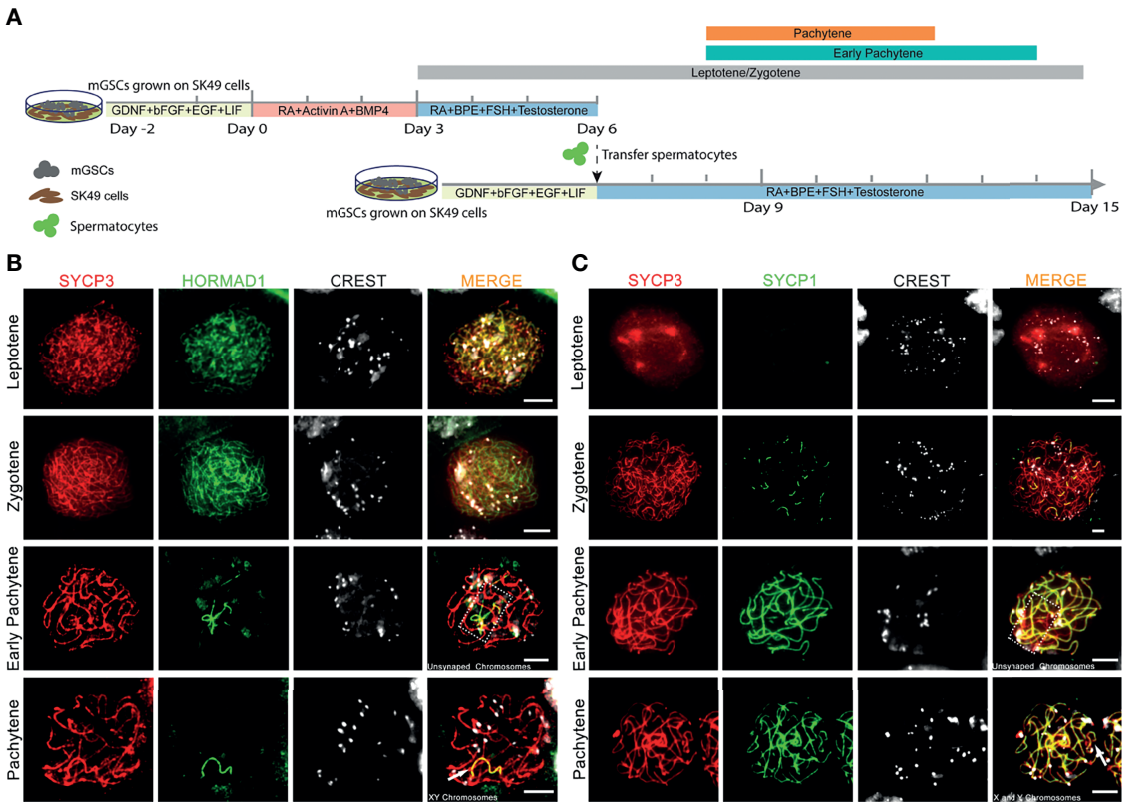

FIGURE 1 | Complete homologous chromosome synapsis during *in vitro* meiosis. **(A)**. Schematic overview of the *in vitro* meiosis culture system. Bars above the timeline represent the period of relatively highly abundant presence of leptotene, zygotene, early pachytene and pachytene spermatocytes. **(B, C)** Unsynapsed chromosomes marked with HORMAD1 **(B)**, and synapsed chromosomes marked with SYCP1 **(C)** in *in vitro*-generated spermatocytes stained for SYCP3 (red), centromeres (CREST, white), HORMAD1 (green) or SYCP1 (green). Unsynapsed chromosomes and X and Y chromosomes are shown in dashed box or by arrowhead respectively. Scale bars, 5μm.

and 15 (**Figure 1A**). To monitor synapsis of the homologous chromosomes, we stained the *in vitro*-generated spermatocytes using antibodies against SYCP3, HORMAD1 and CREST serum to mark the synaptonemal complex, unsynapsed chromosomes and centromeres, respectively. In line with the *in vivo* situation, in which HORMAD1 specifically accumulated on unsynapsed chromosome axes (**Supplementary Figure S1A**) (32), we observed that, *in vitro*, HORMAD1 co-localized with SYCP3 during the leptotene to zygotene stages and disappeared from the axial elements as chromosome synapsis proceeded, ultimately only staining the unsynapsed X and Y chromosomes at full pachynema (**Figure 1B**). We also stained for SYCP1, which is a central element protein of the synaptonemal complex and thus specifically marks synapsed areas of meiotic homologous chromosomes (33). Indeed, we observed no SYCP1 staining during leptonema, and appearance of several short SYCP1 fibers during zygonema. While at the early pachytene stage, some axial elements marked by SYCP3 still lacked SYCP1, all SYCP3, except on the sex chromosomes, fully co-localized with SYCP1 in *in vitro*-generated pachytene spermatocytes (**Figure 1C**). Such pachytene spermatocytes were mostly observed from day 8 to day 12 and were only occasionally present at day 14 (**Figure 1A**). In addition, early pachytene spermatocytes were observed from day 8 to day 14, and leptotene/zygotene spermatocytes from day 3 to day 15 (**Figure 1A**). Hence, in the current system for *in vitro* meiosis, full synapsis of the homologous chromosomes was achieved from day 8 to day 12 after induction of meiosis.

XY Body Formation *In Vitro*

When meiotic synapsis between the autosomes is being completed, the X and Y chromosomes are transcriptionally silenced in a condensed chromatin area called the XY body (34). To investigate whether *in vitro*- generated pachytene spermatocytes also form an XY body, we used antibodies against the DNA damage response proteins γH2AX, MDC1 and ATR that are known to be restricted to the XY body at the pachytene stage *in vivo* (35). γH2AX marks DNA double-strand breaks (DSBs) that are continuously being generated on unsynapsed meiotic chromosomes (36). As a binding partner of γH2AX, mediator of DNA damage checkpoint 1 (MDC1) initiates meiotic sex chromosome inactivation (MSCI) and mediates XY body formation (37). As described by us (17), *in*

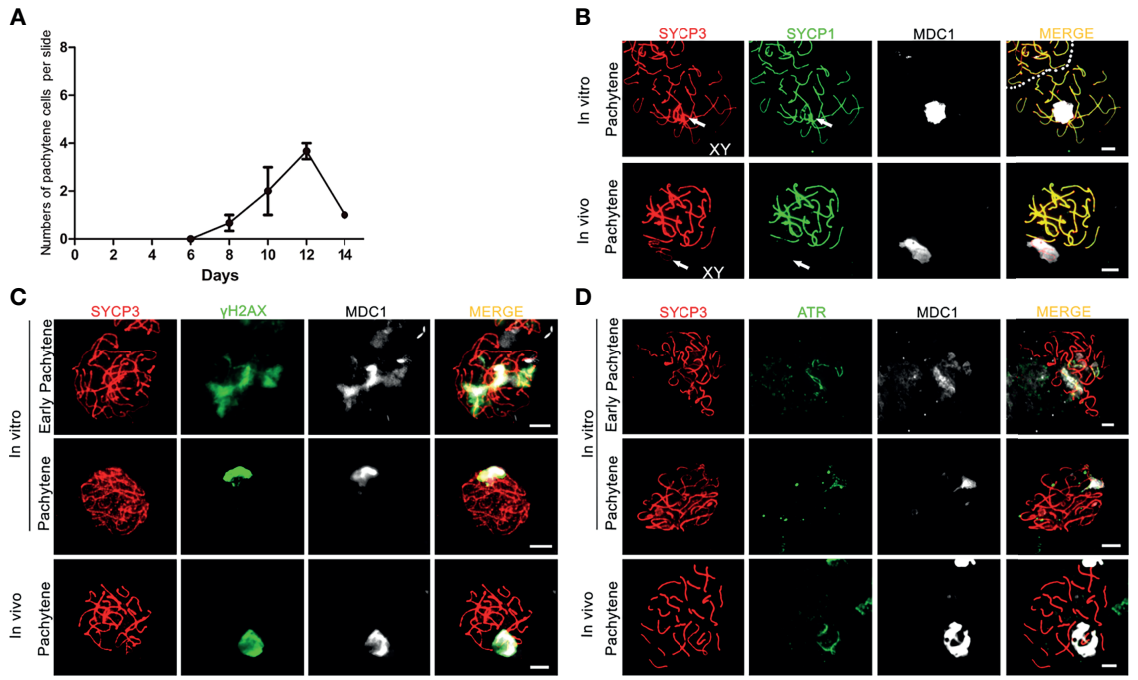

FIGURE 2 | XY body formation during *in vitro* meiosis. **(A)** Number of *in vitro* generated-pachytene cells per microscopy slide. Quantification for each time point was performed using 3 slides from 3 independent experiments. Data are presented as the mean ± SEM. **(B–D)** The XY bodies are marked with **(B)** MDC1, **(C)** γH2AX and **(D)** ATR in *in vitro*- generated early pachytene and pachytene spermatocytes stained for SYCP3 (red) and MDC1 (white), SYCP1(green), γH2AX (green), or ATR (green). *In vivo* pachytene spermatocytes are used for a positive control. Scale bars, 5μm.

vitro-generated pachytene-like cells in our pervious culture system did not display full chromosome synapsis, and thus γH2AX staining remained present on many autosomes while no clear XY body could be discerned. However, in the current culture system, 3 to 4 clear pachytene cells per microscope slide, from day 8 to day 14 (**Figure 2A**), were observed to reach full synapsis of the autosomes, marked by SYCP1 (**Figure 2B**), while clear XY bodies were marked by MDC1 (**Figure 2B**) or γH2AX and MDC1 (**Figure 2C**), which is consistent with pachytene spermatocytes *in vivo* (**Figures 2B, C**). Because the undifferentiated GSCs present in the second culture dish will also initiate meiosis after the change to medium III upon addition of the first generation of meiotic cells, the pachytene spermatocytes (derived from the first generation of meiotic cells) are massively outnumbered by early meiotic cells (derived from these GCSs). We therefore quantified the total numbers of these cell types, and meiotic M-phase cells to record the dynamics of spermatocyte numbers during the culture period, as presented in **Supplementary Figure S2A**.

Similar to γH2AX and MDC1, the ataxia telangiectasia and Rad3-related protein (ATR) also localizes to unsynapsed chromosomes and the XY body *in vivo* (**Figure 2D**), where it is involved in MSCI (38). In our culture system, while still being present on some unsynapsed chromosomes during early pachynema, ATR-staining now, together with MDC1, appeared restricted to the sex chromosomes in *in vitro*-generated pachytene cells (**Figure 2D**). Hence, the current *in vitro* meiosis system supports full synapsis of the homologous chromosomes and formation of the XY body.

Meiotic Recombination Is Not Completed *In Vitro*

In vivo, meiotic DSBs are repaired *via* meiotic recombination, which eventually leads to formation of meiotic crossovers between homologous chromosome pairs (39, 40). The number of initial DSB repair sites, marked by DNA repair protein RAD51, has a peak at leptonema, after which it starts to decline from zygotene until about 1-2 meiotic crossovers, marked by the MutL homolog 1 protein MLH1, remain at the late pachytene stage (40). In contrast to *in vivo* pachytene spermatocytes, in which RAD51-foci were restricted to the X and Y chromosomes, we observed that RAD51-foci remained present on almost all autosomes in *in vitro*-generated pachytene spermatocytes, even when the XY body, marked by MDC1, was already formed (**Figure 3A**).

Finally, we used antibodies against MLH1 to investigated whether meiotic crossovers were formed *in vitro*. In addition, we used antibodies against SYCP1 to mark synapsed homologous chromosomes and MDC1 to mark the XY body. In comparison to the *in vivo* control, which nicely showed 1-2 MLH1 foci on every synapsed chromosome pair, only few meiotic crossovers were

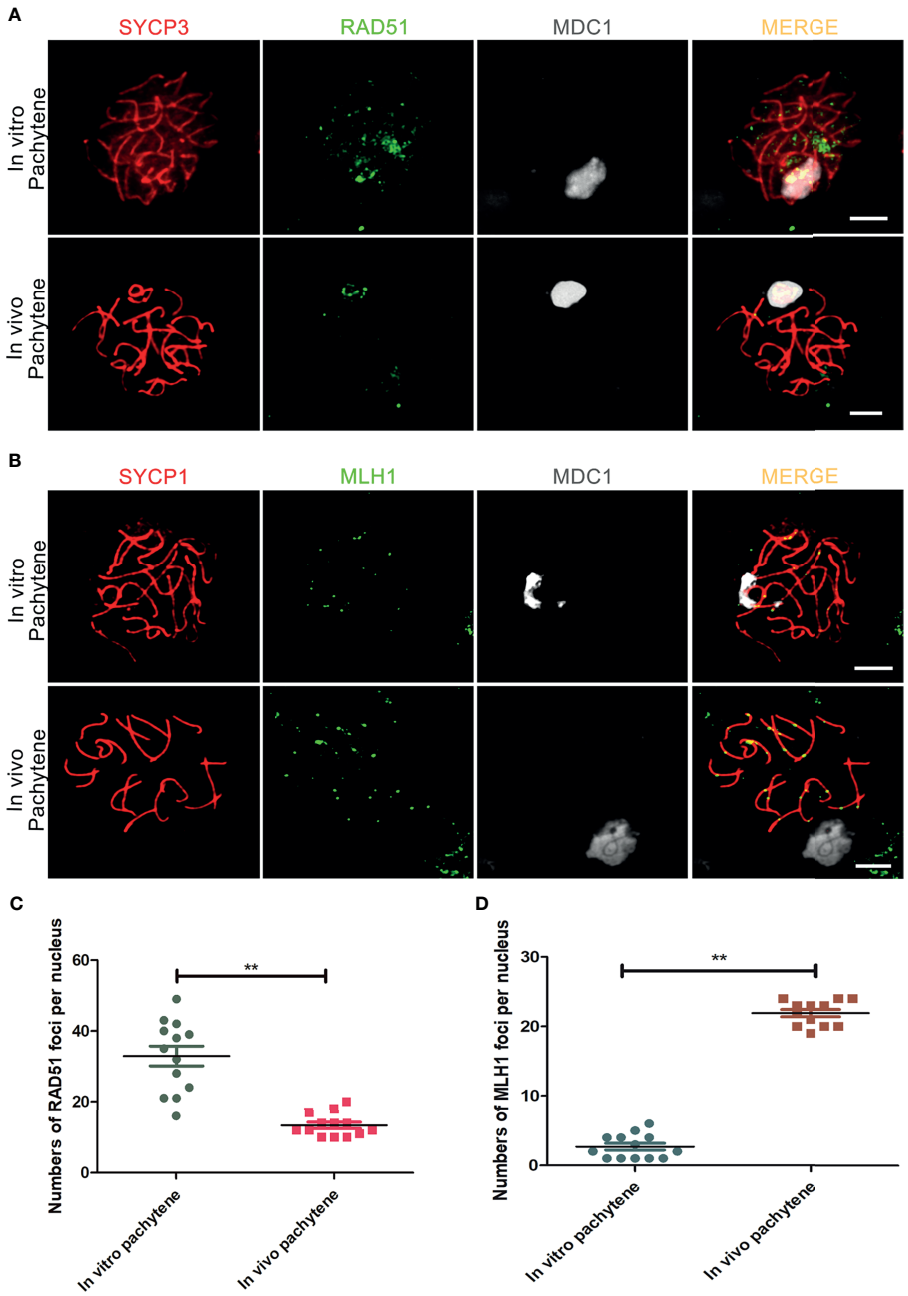

FIGURE 3 | Incomplete meiotic recombination and inefficient meiotic crossover formation *in vitro*. **(A)** DSBs-repair sites, marked with RAD51, are not completely resolved in *in vitro*- generated pachytene spermatocytes stained for SYCP3 (red), RAD51 (green) and MDC1 (white). *In vivo* pachytene spermatocytes are used for a positive control. **(B)** Meiotic crossovers marked with MLH1 in *in vitro* - generated pachytene spermatocytes stained for SYCP1(red), MLH1 (green) and MDC1 (white). *In vivo* pachytene spermatocytes are used for a positive control. Scale bars, 5μm. **(C, D)** Quantification of RAD51-foci per cell nucleus of *in vitro* pachytene spermatocytes (n=13) and *in vivo* pachytene spermatocytes (n=13) and **(D)** Quantification of MLH1-foci per cell nucleus of *in vitro* pachytene spermatocytes (n=13) and *in vivo* pachytene spermatocytes (n=12). Data are presented as the mean ± SEM. **$p < 0.01$ (Student's t-test).

visible in *in vitro*-generated pachytene spermatocytes (**Figure 3B**). For quantification, the number of RAD51 and MHL1 foci in pachytene spermatocytes was counted, showing an increase in RAD51 and a decrease in MLH1 foci *in vitro*, indicating deficient meiotic recombination *in vitro* (**Figures 3C, D**).

Meiotic crossovers are required for the formation of chiasmata, physical links between the homologous chromosome pairs that ensure proper segregation of the homologous chromosomes during the first meiotic M-phase (15). In the current culture system, 1-2 M-phase cells per microscope slides were observed between day 8 to

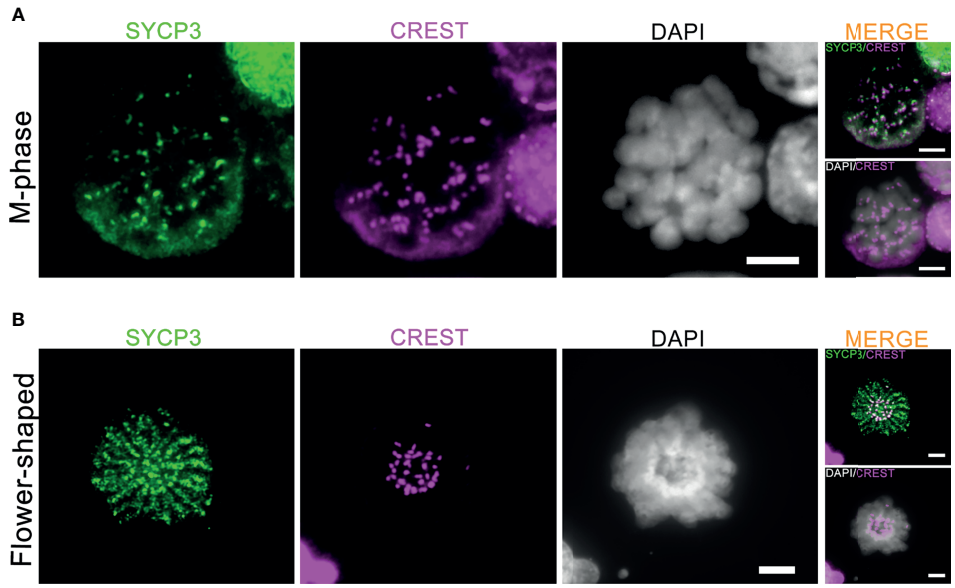

FIGURE 4 | Generation of meiotic M-phase cells and flower-shaped *in vitro*. The co-immunofluorescent staining of SYCP3 (red), centromeres (CREST, pink) and DNA (DAPI, white) for *in vitro*-generated meiotic M-phase cells **(A)** and "flower-shaped" cells **(B)**. Scale bars, 5μm.

day 14 after meiotic induction (**Supplementary Figure S2A**). In accordance with the very inefficient formation of meiotic crossovers, these M-phase cells only displayed pairs of sister chromatids (univalents) with CREST-stained centromeres located at the ends of the paired sister chromatids (**Figure 4A**). In addition, 1-2 flower-shaped cells per microscope slide, identified as a type of premature M-phase cells in our recent study (17), could still be observed between day 8 to day 15 in this current culture system (**Figure 4B**). In all, we conclude that, despite the completion of synapsis and XY body formation, meiotic crossover formation was still not completed *in vitro*, which prevents formation of bivalents (pairs of homologous chromosomes) during the first meiotic M-phase.

Fresh Sertoli Cells Alone Can Support *In Vitro* Chromosome Synapsis

To investigate whether addition of mGSCs is required for synapsis and XY-body formation, we next compared the addition of undifferentiated mGSCs on a freshly plated layer of proliferatively inactivated SK49 Sertoli cells (group A) to a fresh layer of SK49 Sertoli cells alone (group B). Again, we used antibodies against SYCP3, HORMAD1, SYCP1 and MDC1 to mark the synaptonemal complex, unsynapsed chromosomes, synapsed chromosomes and XY body formation respectively. From day 8 to day 12 we observed comparable numbers of pachytene spermatocytes that displayed fully synapsed homologous chromosomes and XY-body formation in both groups (**Figures 5A, B** and **Supplementary Figure S2B**). Moreover, to assess meiotic crossover formation between the both groups, we used antibodies against SYCP1, MLH1 and MDC1 to mark synapsed chromosomes, meiotic crossover and XY body respectively. Again, and in both groups, only very few meiotic crossovers could be observed (**Figure 5C**). In all, omission of fresh mGSCs did not affect meiotic synapsis and XY-body formation in this culture system.

DISCUSSION

As described previously (17), *in vitro* cultured mouse spermatogonia are able to enter meiosis and reach the meiotic M-phase stages and, occasionally, form spermatid-like cells. However, the *in vitro*-generated pachytene spermatocytes displayed incomplete synapsis of the homologous chromosomes, did not form an XY body and did not form meiotic crossovers. As a result, this led to meiotic MI-phase cells with univalent chromosomes (pairs of sister chromatids), instead of bivalents (pairs of homologous chromosomes). Usually, *in vivo*, meiotic prophase checkpoints eliminate pachytene spermatocytes with unsynapsed chromosomes, aberrant XY body formation or remaining unrepaired meiotic DSBs (12), preventing such spermatocytes to progress to the metaphase stage. Apparently, these meiotic prophase checkpoints were not functionally activated in our previous culture system. We here aimed to optimize our *in vitro* culture system so that it supports full meiotic chromosome synapsis, XY-body formation and DSB- repair.

In vivo, multiple layers of germ cells and Sertoli cells are well-organized in the seminiferous tubules of testis, enabling the interactions of different testicular cells that support complete spermatogenesis. For instance, retinoic acid does not only induce spermatogonial differentiation and meiotic initiation; once germ cells have entered meiosis, pachytene spermatocytes also produce RA to coordinate spermatogenesis (41). The current culture system, in which *in vitro*-generated spermatocytes are transferred to a

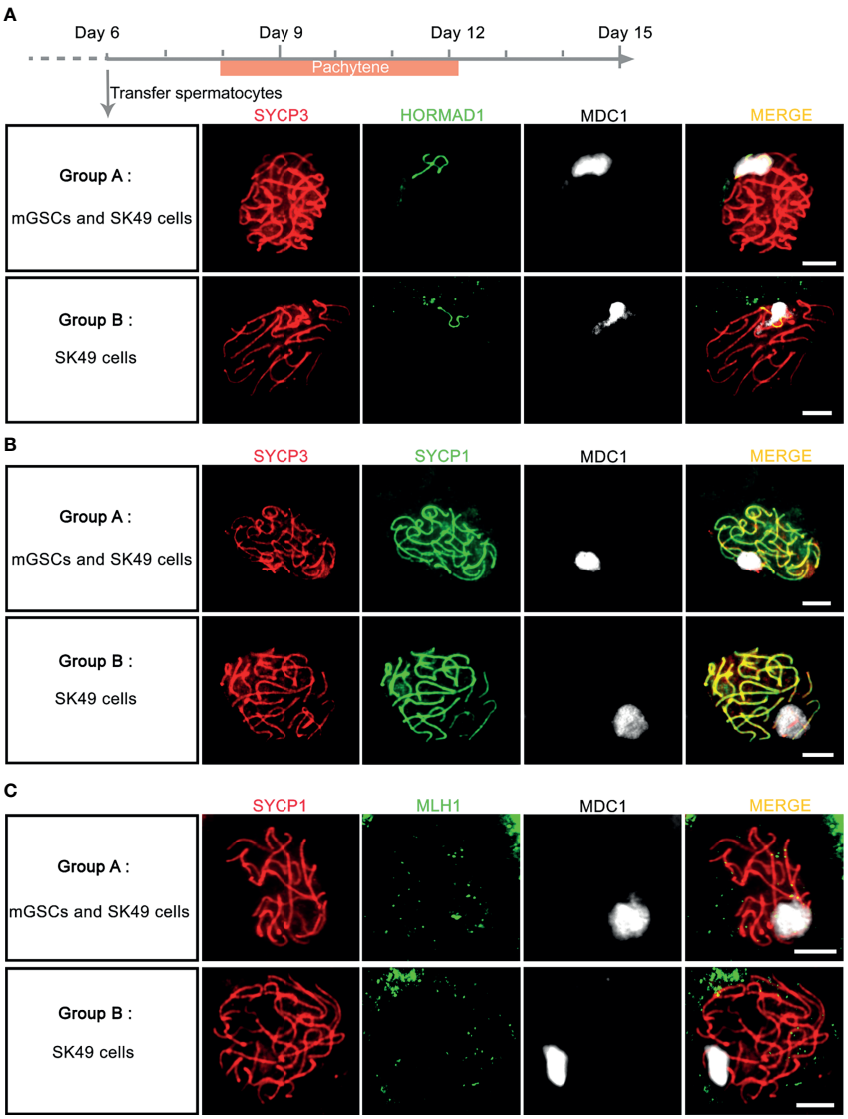

FIGURE 5 | Chromosome synapsis was completed without the addition of fresh mGSCs. **(A, B)** *In vitro* - generated pachytene spermatocytes stained for SYCP3 (red), MDC1 (white), **(A)** HORMAD1 (green) or **(B)** SYCP1 (green) were observed both group A (undifferentiated mGSCs on a fresh layer of proliferatively inactivated SK49 Sertoli cells) and group B (a fresh layer of proliferatively inactivated SK49 Sertoli cells alone) from day 8 to day 12. **(C)** *In vitro*- generated pachytene spermatocytes stained for SYCP1 (red), MLH1 (green) and MDC1 (white) in group **(A, B)**. Scale bars, 5μm.

subsequent culture dish containing fresh spermatogonia and Sertoli cells, was designed to more closely resemble the *in vivo* situation in which germ cells at different developmental stage co-exist. Although we did find pachytene spermatocytes with complete meiotic chromosome synapsis and XY-body formation in the current culture system, the number of pachytene spermatocytes appeared low due to cell loss during the transfer of spermatocytes to the second dish. The absence of spermatid-like cells may also be due to this loss in transfer. Interestingly, also transfer of *in vitro*-generated spermatocytes to a plate containing only freshly plated Sertoli cells supported complete meiotic chromosome synapsis and XY-body formation. Although only performed once in parallel with the current culture system presented here, we never found complete meiotic chromosome synapsis in our previous culture system (17). Hence, independent of the presence of additional germ cell types, transfer of developing meiotic cells to a fresh monolayer of Sertoli cells appears to be essential for proper meiotic chromosome synapsis.

Still, also in the improved culture system, only very few meiotic crossovers were formed leading to meiotic MI cells with univalent instead of bivalent chromosome pairs. Usually, *in vivo*, meiotic chromosome synapsis and recombination are two highly intertwined processes. Homologous chromosome synapsis is required for DSB-repair sites to develop into meiotic crossovers and incomplete synapsis will lead to incomplete DSB-repair and

subsequent failure of crossover formation (33). On the other hand, the initiation of synapsis requires the introduction of DSBs (13, 42), impaired meiotic DSB repair causes aberrant chromosome synapsis and subsequent meiotic prophase arrest (43, 44). However, during *in vitro* meiosis, we observed that, although chromosome synapsis was complete, still very few crossovers were detected. The reason may be that some DSB sites were not repaired timely and remained present at synapsed chromosomes. Indeed, in our *in vitro* pachytene spermatocytes, DSB-repair sites marked by RAD51 did not disappear completely from the autosomal chromosomes. Another reason why still so few crossovers were formed could be that too many DSBs may be repaired as non-crossover products *in vitro*. Also *in vivo*, only a small fraction of all DSBs are repaired as crossover products, although still leading to at least one or two crossovers per homologous chromosome pair, while the remaining DSBs give rise to non-crossovers (45). This balance between crossover and non-crossover formation may be off *in vitro*.

The lack of influence of co-cultured spermatogonia on meiotic progression could be due to the fact that they themselves quickly differentiate and become early spermatocytes. For future investigations, undifferentiated spermatogonia that are incapable of differentiation, for instance *c-kit* knockout spermatogonia, could be used to ensure continued co-culture with mitotic germ cells. In addition, in the improved culture system, the *in vitro*-generated germ cells can be cultured up to 14 days, which is longer than our previous culture system and close to the *in vivo* situation in which the mouse meiotic prophase normally takes about 2 weeks (46). However, unlike the *in vivo* situation, the pachytene spermatocyte numbers are still relatively low, and most of meiotic cell types are leptotene and zygotene spermatocytes during the entire culture period.

The presence of meiotic MI-phase cells with univalent chromosomes and flower-shaped cells, which entered the meiotic M-phase prematurely (17), indicates that meiotic checkpoints are still not fully functional in the current system. To form bivalent chromosome pairs at MI proper meiotic recombination and crossover formation are required and *in vivo* spermatocytes that fail to do so are eliminated by these checkpoints (12). Even though meiotic crossovers and meiotic checkpoints are there to ensure correct chromosome segregation, meiotic crossovers and subsequent chiasmata formation have been described only rarely by previous *in vitro* spermatogenesis studies or studies using *ex vivo* cultures of testicular tissues. To our knowledge, only one study described the formation of chiasmata and bivalent chromosome pairs in *in vitro*-generated MI-spermatocytes, however, the meiotic recombination process and subsequent crossover formation was not described (2). Thus, many details about meiotic DSB-repair and crossover formation during *in vitro* meiosis remain largely unknown. Considering the fact that *in vitro*-generated gametes may be clinically used in the future, investigation of meiotic DSB repair and crossover formation, and the checkpoints that monitor these processes, should be included in the characterization of novel *in vitro* gametogenesis protocols. Only then can generation of gametes without unpaired DSBs or aneuploid sets of chromosomes be guaranteed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by animal ethical committee of the Amsterdam UMC, Academic Medical Center, University of Amsterdam.

AUTHOR CONTRIBUTIONS

QL, AP, and GH designed the experiments. QL and EZ performed the experiments. QL and GH analyzed the data. QL, AP, and GH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.761249/full#supplementary-material

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Long-Term Maintenance and Meiotic Entry of Early Germ Cells in Murine Testicular Organoids Functionalized by 3D Printed Scaffolds and Air-Medium Interface Cultivation

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Short-term germ cell survival and central tissue degeneration limit organoid cultures. Here, testicular organoids (TOs) were generated from two different mouse strains in 3D printed one-layer scaffolds (1LS) at the air-medium interface displaying tubule-like structures and Leydig cell functionality supporting long-term survival and differentiation of germ cells to the meiotic phase. Chimeric TOs, consisting of a mixture of primary testicular cells and EGFP$^+$ germline stem (GS) cells, were cultured in two-layer scaffolds (2LSs) for better entrapment. They showed an improved spheroidal morphology consisting of one intact tubule-like structure and surrounding interstitium, representing the functional unit of a testis. However, GS cells did not survive long-term culture. Consequently, further optimization of the culture medium is required to enhance the maintenance and differentiation of germ cells. The opportunities TOs offer to manipulate somatic and germ cells are essential for the study of male infertility and the search for potential therapies.

Keywords: organoid, 3D printing, testis, spermatogonial stem cells, germline stem cells, tubulogenesis, *in vitro* spermatogenesis

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INTRODUCTION

Spermatogenesis is the stepwise process of sperm development within the seminiferous tubules of the testis. The tubules are delimited by a basement membrane and contractile myoid cells. In the tubular compartment, Sertoli cells directly nurture germ cells through the different stages of spermatogenesis: from spermatogonial stem cell (SSC) at the basement membrane to post-meiotic spermatids toward the lumen. The fate of germ cells also relies on secretions coming from the interstitial compartment between the tubules such as testosterone produced by the Leydig cells (Oliver and Stukenborg, 2019). Because of the highly coordinated nature of signaling cues in the testicular microenvironment, it is pivotal for cultures for *in vitro* spermatogenesis (IVS) to reestablish conditions that mimic the two structural compartments of the testis (Oliver and Stukenborg, 2019).

Organ cultures of testicular tissue fragments have been successfully used for IVS in rodents because they best preserve the SSC niche (Sato et al., 2011). However, they do not offer the ability to access and manipulate single cells, making it an inefficient approach for mechanistic studies *ex vivo*. In contrast, testicular organoids (TOs) are customizable and therefore permit detailed analysis of mechanisms underlying testis development and function (Alves-Lopes and Stukenborg, 2018). TOs are 3D multicellular testis surrogates originating from the assembly of cells in suspension. Their fabrication mostly relied on 3D scaffolds made of extracellular matrix (ECM) because of the provision of instructive and structural support to the reorganizing cells (Richer et al., 2020). For instance, tubulogenesis was reported in collagen and Matrigel-based scaffolds, which were not able to support complete spermatogenesis (Zhang et al., 2014; Alves-Lopes et al., 2017). Moreover, the animal and carcinogenic origin of commercially available matrices impedes their clinical application. To overcome these limitations and more closely mimic the native testicular tissue, decellularized testicular matrices were employed (Baert et al., 2017; Pendergraft et al., 2017; Topraggaleh et al., 2019; Vermeulen et al., 2019). Still, although testicular cells from piglets reorganized into TOs with compartmentalized tubule-like structures in porcine-derived testicular matrix, complete spermatogenesis was not observed (Vermeulen et al., 2019). Interestingly, 3D ECM-free approaches also resulted in the formation of compartmentalized organoids from mouse testicular cells, challenging the idea that TO formation depends on external matrices (Yokonishi et al., 2013; Sakib et al., 2019; Edmonds and Woodruff, 2020). Edmonds and Woodruff (2020) elegantly examined the requirement for exogenous ECM-support for TO formation from prepubertal mouse testicular cells in both 2D and 3D immersion culture conditions. In their study, testicular cell self-assembly and self-reorganization were optimal in 3D ECM-free culture and, thus, relied solely on the intrinsic morphogenic capacity of the dissociated immature cells themselves. It was suggested that non-cellular material in the ECM-based culture may have hindered intercellular interactions (Edmonds and Woodruff, 2020). However, germ cells were rarely observed after 14 days in culture (Edmonds and Woodruff, 2020). In contrast, Yokonishi et al. (2013) were able to maintain primary germ cells and germline stem (GS) cells (precultured SSCs) for 61 days and 8 weeks, respectively, in tubule-like structures of millimeter-sized TOs at the air-medium interface (Yokonishi et al., 2013). Noteworthy, cultivation of large tissues imposes the risk of central degeneration, typically occurring in organ culture because of insufficient nutrient supply and anoxia (Kojima et al., 2018). Meanwhile, the use of too low testicular cell densities was shown to result in small spheroidal-shaped aggregates having a reversed architecture (inside-out) or even completely lacking a genuine testicular architecture, likely due to limited intercellular interactions coming from excessive miniaturization (Baert et al., 2019; Sakib et al., 2019).

To restore testicular architecture and avoid degeneration of the organoid core in order to ultimately improve IVS, the current study aimed at forming TOs from prepubertal mouse testicular cells at the air-medium interface in defined 3D printed macropores. The macroporous scaffolds solely served as a delimitation of the area in which the cells can reorganize, with the purpose of scaling the TOs to one functional unit of the testes, representing a seminiferous tubule with surrounding interstitium. Moreover, strain-dependent differences of testicular cells to reorganize and function were examined, as these features affect the efficiency of IVS in organ culture (Portela et al., 2019). Lastly, the possibility to grow GS cells in TOs was investigated, as their use would extend the applicability of TOs in research and development.

MATERIALS AND METHODS

Mice

Testes were obtained from prepubertal wild-type C57BL/6 J or hybrid CBAB6F1 pups (4–5 days old) from the institutional breeding facility (LA2230395: Vrije Universiteit Brussel; SABL063: Monash Animal Research Platform). Following isolation and mechanical removal of the tunica albuginea, testes were cryopreserved by uncontrolled slow freezing as previously described (Baert et al., 2012). Transgenic GS cells were derived from newborn Tg(CAG-EGFP)131Osb males according to an established method (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara, 2005). In these mice, the EGFP transgene is driven by the cytomegalovirus intermediate early enhancer coupled to the chicken beta actin promoter, resulting in ubiquitous expression of a green fluorescent reporter. Experimental procedures and animal breeding were approved by the Ethical Committee for the use of Laboratory Animals of the Vrije Universiteit Brussel (permission 19-216-1 under license LA1230216) and Monash Medical Centre B Animal Ethics Committee (MMCB/2019/01, under license SPPL20173).

Testicular Cell Isolation

Cryopreserved testes were removed from storage after 1–6 months and thawed for 2 min in a water bath at 37°C. Cryoprotectant was diluted by washing the testes twice in Dulbecco's Modified Eagle's Medium (DMEM)/F12 containing 10% (v/v) fetal calf serum (10500-056; Thermo Fisher, Merelbeke, Belgium). To obtain single cell suspensions, testes were enzymatically digested using 1.0 mg/ml collagenase Ia (C9891; Sigma-Aldrich, Diegem, Belgium), 0.5 mg/ml hyaluronidase (H3506; Sigma-Aldrich), and 0.5 mg/ml desoxyribonuclease (DN25, Sigma-Aldrich) in Minimum Essential Medium-alpha (αMEM, 32571028; Thermo Fisher; Baert et al., 2019). Cell aggregates were removed by filtration with a 40 µm cell strainer (35234; BD Falcon, Leuven, Belgium). After digestion, cell viability was assessed using the 0.4% trypan blue exclusion test (15250061; Thermo Fisher) in a Neubauer chamber (Neubauer, Blaubrand, Germany) and ranged from 78 to 84%. The final cell seeding densities only took the concentrations of viable cells into account.

Germline Stem Cell Culture

The GS cell culture was set up according to a previously established protocol (Kanatsu-Shinohara et al., 2003, 2005).

Briefly, following enzymatic digestion, testicular cell suspensions from newborn Tg(CAG-EGFP)131Osb males were cultured on 0.2% (w/v) gelatin-coated dishes and passaged to remove most somatic cells which adhered to the coated dishes. The floating cells, enriched for GS cells, were cultured on inactivated mouse embryonic fibroblast feeder cells in GS cell medium (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara, 2005). The GS cell cultures were kept at 37°C with 5% CO_2 in air and passaged weekly, depending on the proliferation rate of GS cells (Kanatsu-Shinohara, 2005). Medium change was performed every 2–3 days.

Design and Printing of Scaffolds

A square-shaped one-layer scaffold (1LS) with regularly spaced pores in a grid-like pattern (3.5 mm × 3.5 mm, 0.15 mm height with a strut distance of 1.1 mm, strand thickness of 0.6 mm and angle change of 90°) and a mold (4.2 mm × 4.2 mm squares, 1.5 mm height with no spacing between the struts) were designed with Sketchup software (Trimble, Sunnyvale, United States). 3D printing STL files were repaired with Microsoft's Azure (Microsoft, Redmond, United States) and sliced into a G-code using Heartware software (Cellink, Göteborg, Sweden; **Figure 1A**). Design and slicing of a circular two-layer scaffold (2LS; 7 mm diameter, 0.6 mm height and grid-like pattern with strut distance of 1.3 mm, strand thickness of 0.6 mm and angle change of 90°) were done with GeSIM Robotics v.1.4.0 software (**Figure 1B**). Cellink-RGD bioink (nanocellulose-alginate hydrogel with additional arginine-glycine-aspartic acid cell-attachment motifs) was used for printing 1LSs, molds and 2LSs, while regular Cellink bioink lacking RGD binding motifs (Cellink) was also used for 2LSs to promote confinement of the cells within the macropores. The hydrogels were extruded at room temperature from a 25 gauge conical nozzle (Subrex, Carlsbad, United States) onto glass slides using a pneumatic extrusion-based Inkredible+ bioprinter (Cellink) for 1LSs and the molds or the pneumatic extrusion-based Bioscaffolder (SYSENG, Salzgitter, Germany) for 2LSs. Extruding pressures of the printing process ranged from 5 to 15 kPa and 24 kPa, and the printing speed from 17.5 to 20 mm s^{-1} and 13 to 20 mm s^{-1}, respectively. To allow polymerization of the bioink, the printed hydrogels were crosslinked for 5 min with 100 mM $CaCl_2$ (Cellink), followed by a rinse with αMEM.

Scaffold Preparation

To ensure proper cell seeding inside the printed macropores, 1LSs (3.5 mm^2) were placed inside agarose sockets (Baert et al., 2019). For this, a 0.35% (w/v) gel stand was created by placing 100 µl of molten agarose (16500100; Thermo Fisher) in 24-well plate hanging culture inserts with a diameter of 7 mm (MCRP24H48; Sigma-Aldrich). When agarose had gelled, crosslinked molds (4.2 mm^2) were placed

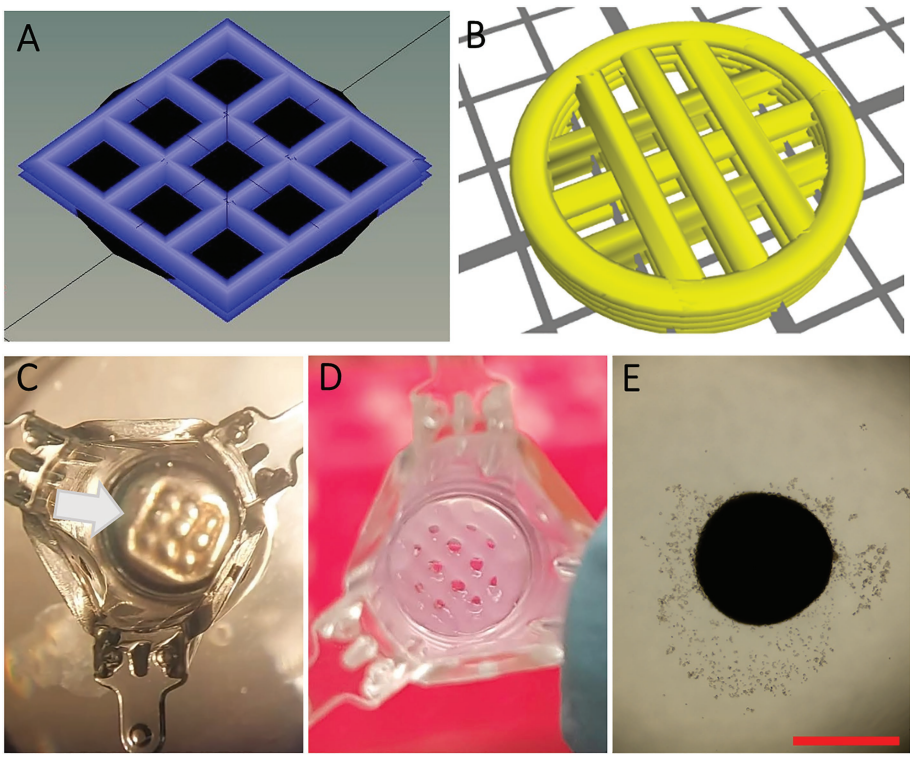

FIGURE 1 | Design and preparation of scaffolds. **(A-B)** 3D design of square-shaped one-layer scaffolds (1LSs, **A**) and circular two-layer scaffolds (2LSs, **B**). **(C)** To ensure proper cell seeding inside its macropores, 1LSs were transferred inside agarose sockets (white arrow) in culture inserts. **(D)** Because of their diameter size, 2LSs were directly transferred in the culture inserts. **(E)** A control condition without 3D printed scaffold was included. Bar = 500 µm.

onto the gel stands. Then, 25 µl of molten agarose was pipetted around the molds and left to harden. The removal of the molds resulted in agarose sockets (**Figure 1C**, white arrow) in which crosslinked 1LSs were positioned using a spatula. Because of their size (7 mm diameter), 2LSs were directly transferred onto agarose gel stands placed in the culture inserts (**Figure 1D**).

Testicular Organoid Culture

TOs were prepared by seeding 1×10^6 primary testicular cells (±three pups) in a 30 µl drop of basal medium onto 1LSs and control agarose stands without scaffold (**Figure 1E**) (C57BL/6J, $n=5$ technical replicates; CBAB6F1, $n=3$;) on one hand, or a chimeric mixture of testicular cells from prepubertal C57BL/6J mice and cultured GS cells onto 2LSs at a ratio of 2:1 ($n=3$ technical replicates per condition) on the other (Yokonishi et al., 2013). Primary and chimeric TOs were cultured for up to 6 weeks at the air-medium interface at 35°C in a humidified atmosphere with a normal O_2 tension (21%) and 5% CO_2. The basolateral compartment of the culture wells was filled with 700 µl basal culture medium consisting of αMEM supplemented with 10% (v/v) KnockOut Serum Replacement (KSR, 10828010; Thermo Fisher), 1% (v/v) Penicillin Streptomycin (1540-122; Thermo Fisher) and 10^{-7} M melatonin (M5250; Sigma-Aldrich), successfully used previously to induce complete spermatogenesis in murine organ and cell culture (Reda et al., 2017; Baert et al., 2019). Additionally, for chimeric TOs during long-term culture, basal medium was supplemented with 10^{-6} M retinol (R7632; Sigma-Aldrich) as precursor of retinoic acid, which has been shown to improve spermatogenesis from SSCs and cultured pluripotent stem cells (Arkoun et al., 2015; Wang et al., 2016). Retinol was added every second day and medium was refreshed and collected weekly for hormone analyses. Gross morphology was evaluated using a stereomicroscope. The graphics depicting the primary TO and chimeric TO culture set ups were created with BioRender.com (RY23APYYXPS).

Immunocytochemistry

Prior to initiation of the TO cultures, testicular cells obtained after the enzymatic digestion of testes from C57BL/6J or CBAB6F1 mice were re-suspended at 1×10^6 cell/ml of cytospin buffer containing phosphate-buffered saline [PBS (1X), 70011051; ThermoFisher] and 1% (w/v) bovine serum albumin (10735094001; Roche). Around 30 µl of the cell suspension was loaded in cytofunnels and centrifuged onto glass slides (J1800AMNZ; VWR) at 1350 rpm for 3 min. Subsequently, the slides were dried at room temperature, fixed for 10 min in 4% formaldehyde at room temperature and subjected to heat-mediated epitope retrieval at 95°C in 10 mM Tris-EDTA buffer (pH 9.0). Following three washes of 5 min in PBS, the slides were blocked with 5% serum and then incubated overnight in a humid chamber at 4°C with mouse monoclonal anti-phospho-histone H2A.X (γH2AX, 05-636-I; Sigma Aldrich, 1:100) and rabbit polyclonal anti-DEAD-box helicase 4 (DDX4, ab13840; Abcam, 1:400) primary antibodies diluted in PBS. The following day, the slides were washed with PBS and incubated for 1 h at room temperature with donkey anti-rabbit 488 (A21206; Life technologies, 1:200) and donkey anti-mouse 594 (A21203; Life Technologies) secondaries in PBS. Following additional PBS washes, the slides were mounted with Prolong Gold Antifade Mountant with DAPI (P36931; Thermo Fisher) and stored at 4°C until evaluation.

Histological and Immunofluorescent Analyses

Following fixation in acidified alcoholic formalin (PFAFA0060AF59001; Labonord), primary TOs in 1LSs were captured in 2% (v/v) agarose, dehydrated to 70% ethanol and embedded in paraffin. Paraffin blocks were sliced into 5 µm thick serial sections (SM2010R; Leica). The slides were deparaffinized in xylene and rehydrated in descending concentrations of isopropanol (100, 100, 90, and 70%). Following a 5 min wash in PBS, the sections were stained with periodic acid-Schiff (PAS) and hematoxylin to perform gross morphological evaluations of the samples. Chimeric TOs were handled differently: after 1 or 6 weeks of culture, they were fixed with 4% formaldehyde in PBS and cryo-embedded in optimal temperature cutting compound to preserve the EGFP signal. Eight µm thick serial frozen sections were stained with Masson's trichome for representative images of the samples. For in-depth analysis of primary and chimeric TOs, immunofluorescent staining was performed to detect key phenotypical and functional testicular markers: laminin (LN, marks basement membrane), zona occludens 1 (ZO1, tight junctions), 3beta-hydroxysteroid dehydrogenase (3ß-HSD, Leydig cells), actin alpha 2 (ACTA2, myoid cells), SRY-Box transcription factor 9 (SOX9, Sertoli cells), DDX4 (germ cells), γH2AX (meiotic spermatocytes), cAMP-responsive element modulator (CREM, spermatids), and peanut agglutinin (PNA, acrosome). Detailed protocols are listed in **Table 1**. Briefly, slides were subjected to epitope retrieval at 95°C in 10 mM Tris-EDTA buffer (pH 9.0), followed by three washes of 5 min in PBS and then blocked with 5% serum in PBS at room temperature. The slides were then incubated overnight in a humid chamber with primary antibodies diluted in PBS at 4°C, and the following day, washed with PBS and incubated with the secondary antibodies diluted in PBS. Following additional PBS washes, the slides were mounted with Prolong Gold Antifade Mountant with DAPI or SYTOX Deep Red Nucleic Acid Stain (P36987; Thermo Fisher) and stored at 4°C until evaluation. For the identification of apoptotic cells, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, *In Situ* Cell Death Detection Kit, Fluorescein, 11684795910; Roche) assay was performed according to the manufacturer instructions with an enzyme solution diluted 1:10 in the TUNEL reaction mix. Testis sections from adult mice were used as positive controls for the testicular markers, while sections treated with 1,500 U/ml DNase I recombinant were used as positive controls for the TUNEL assay. As negative controls, samples were incubated with PBS or the TUNEL reaction mix only, omitting the primary antibodies or the terminal deoxynucleotidyl transferase enzyme solution, respectively. Representative bright field and fluorescence images were taken on an inverted Olympus or Leica microscope. ImageJ software (National Institute of Health) was used to stack and analyze the images. In addition, germ cell

TABLE 1 | Antibody information and reagents used for immunofluorescent stainings.

Protein	Target	Washing buffer (3 × 5 min at room temperature)	Epitope retrieval 75 (paraffin-embedded) or 30 (frozen) min at 95°C]	Blocking (1 h at room temperature)	Primary antibody (overnight at 4°C)	Primary antibody type	Primary antibody dilution	Secondary antibody (1 h at 4°C)
SOX9	Sertoli cells	0.05% Tween-20 in PBS	TE buffer[a]	5% NDS[b]	AB5535[c]	Rabbit pAb	1:200	Donkey anti rabbit 488[g]
ACTA2	Myoid cells	PBS	TE buffer	5% NDS	A2547[d]	Mouse mAb	1:400	Donkey anti mouse 594[g]
γH2AX	Meiotic cells	PBS	TE buffer	5% NDS	05-636-I[d]	Mouse mAb	1:100	Donkey anti rabbit 594[g]
3β-HSD	Leydig cells	PBS	TE buffer	5% NDS	15516-1-A[e]	Rabbit pAb	1:50	Donkey anti rabbit 488[g]
DDX4	Germ cells	PBS	TE buffer	5% NDS	AF2030[f]	Goat pAb	1:100	Donkey anti goat 647[g]
DDX4	Germ cells	PBS	TE buffer	5% NDS	ab13840[g]	Rabbit pAb	1:400	Donkey anti rabbit 488
ZO1	Tight junctions	PBS	TE buffer	5% NDS	617300[h]	Rabbit pAb	1:200	Donkey anti rabbit 488
LN	Laminin	PBS	TE buffer	5% NDS	ab11575[g]	Rabbit pAb	1:100	Donkey anti rabbit 488
CREM	Post-meiotic cells	PBS	TE buffer	5% NDS	sc-440[i]	Rabbit pAb	1:400	Donkey anti rabbit 488

[a] *10 mM Tris base, 1 mM EDTA solution, 0.05% Tween-20, and pH 9.0.*
[b] *Jackson ImmunoResearch Europe Ltd., Sufford, United Kingdom.*
[c] *Millipore, Overijse, Belgium.*
[d] *Sigma-Aldrich, Diegem, Belgium.*
[e] *Proteintech, Manchester, United Kingdom.*
[f] *R&D systems, Abingdon, United Kingdom.*
[g] *Abcam, Cambridge, United Kingdom.*
[h] *Thermo Fisher, Merelbeke, Belgium.*
[i] *Santa Cruz, Heidelberg, Germany.*

differentiation inside tubule-like structures of primary TOs was analyzed with an Axio Scan Z.1 slide scanner (Carl Zeiss) and Zen Lite software (Carl Zeiss).

Functionality of Leydig Cells

Concentrations of testosterone were assessed with the Elecsys Testosterone II competitive immunoassay in a Cobas 8000 bioanalyzer (Roche Diagnostics). Briefly, 20 µl of the sample was incubated with a biotinylated monoclonal testosterone-specific antibody. Streptavidin-coated microparticles and a testosterone derivative labeled with a ruthenium complex were added in order to bind the formed complex to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring wells, where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell/ProCell M (Roche Diagnostics). Hormone concentrations were assessed by electrochemiluminescence, measured by a photomultiplier with a functional sensitivity of the assay of 0.120 ng/ml.

Quantification of Germ Cell Types

Quantification of γH2AX and DDX4$^+$ germ cells was performed prior to culture (day 0) and week 6 of culture on cytospins and TO sections, respectively. Pre-meiotic (DDX4$^+$/γH2AX$^-$) and meiotic (DDX4$^+$/γH2AX$^+$) leptonema and zygonema were identified based on the cytoplasmic expression of DDX4 and the nuclear distribution of γH2AX. Three representative fields per cytospin slide (CBAB66F1 and C57BL/6J: $n = 3$) were imaged at 20X on an inverted Olympus microscope. ImageJ software (National Institute of Health) was used to stack and count the amount of positive and negative cells on each image. For the TOs, independent cross-sections (minimum 25 µm apart) of every technical replicate (CBAB66F1: $n = 3$; C57BL/6J: $n = 5$) were analyzed with Zen Lite software (Carl Zeiss). The percentages of calculated germ cells per strain were plotted in graphs for each time point. Additionally, the results were expressed as the ratio between the number of DDX4$^+$ germ cells in the newly formed tubule-like structures and the total tubule area (mm^2) of the organoids, per strain. Finally, the relative percentages of germ cell populations (pre-meiotic, leptonema, zygonema) in the TOs were calculated.

Statistical Analyses

Statistical analyses were performed using Prism software (GraphPad Prism 8). The influence of culture period and mouse strain on germ cell numbers were determined by mixed-effects analysis followed by Sidak's multiple comparisons test. The influence of mouse strain on germ cell numbers/tubular area was determined by Mann-Whitney test. Concentrations of testosterone over time and between the mouse strains at weekly timepoints were analyzed using a repeated measures two-way ANOVA followed by a Tukey's *post hoc* test. Results are presented as single dots, mean and SD. Statistical significance was set at $p < 0.05$.

RESULTS

Primary TOs From Two Different Mouse Strains Display Similar Characteristics During Long-Term Culture in Cellink-RGD 1LS

1LS-TOs Exhibit a Compartmentalized Testicular Architecture With Re-establishment of the SSC Niche Components, but Also a Heterogeneous Morphology and Inconsistent Tissue Health

When primary testicular cells were cultured on agarose stands without delimitation, they self-reorganized into structures

without size restrictions (**Figure 1E**). To delimitate the area in which testicular cells reorganize, macroporous (pore diameter of 473,8 ± 99,2 µm) 1LSs were printed composed of cell-interactive Cellink-RGD (**Figures 1A,C, 2A**; Baert et al., 2019). After 6 weeks in basal medium, testicular cells from both mouse strains had self-reorganized into compartmentalized organoids with one or more distinct tubule-like structures with lumen, epithelium, and tubular wall, surrounded by an interstitium (**Figures 2B,C**). These TOs were healthy in the sense that there were no signs of focalized cell death (**Figure 2D**, left panel). However, when cells from adjacent pores aggregated, large TOs displaying signs of core degeneration were formed (**Figure 2D**, right panel; **Supplementary Figure 1A**). Tubule-like structures of TOs in 1LSs from both strains displayed similar somatic marker spatial arrangements, resembling their *in vivo* expression profiles (**Supplementary Figures 1B–E**): Leydig (3ß-HSD$^+$) and elongated peritubular myoid (ACTA2$^+$) cells reorganized around the basement membrane (LN$^+$) of tubules containing Sertoli cells (SOX9$^+$) that were interconnected by tight junctions (ZO1$^+$; **Figures 2E–H**).

1LS-TOs Enable Long-Term Survival and Meiotic Entry of Early Germ Cells

Germ cell differentiation in the tubule-like structures of primary TOs was assessed by triple immunofluorescence staining for DDX4, CREM, and PNA at week 6. *In vivo*, DDX4 is localized in the cytoplasm of germ cells, with the strongest signal at the spermatocyte stage (**Supplementary Figure 1F**, red arrowhead). While CREM protein is localized in nuclei, PNA binds to components of the acrosomal matrix, specifically in the cytoplasm of round (DDX4$^+$/CREM$^+$/PNA$^+$: **Supplementary Figure 1F**, red arrow) and elongated (DDX4$^-$/CREM$^+$/PNA$^+$: **Supplementary Figure 1F**, red triangle, inset) spermatids. Although no post-meiotic germ cells could be observed as indicated by the absence of CREM and PNA expression, 26 ± 33 (C56BL/6J) and 20 ± 14 (CBAB6F1) DDX4$^+$ germ cells per mm^2 tubular area could still be observed at the basement membrane but also at the apical side of the epithelium, suggesting the presence of meiotic spermatocytes in both strains (**Figure 2I**, orange arrow; **Figures 3A,B**). Therefore, the expression of DDX4 and nuclear distribution of γH2AX was used to define the meiotic stage to which spermatocytes had progressed. *In vivo*, leptotene spermatocytes are represented by faint DDX4 and bright γH2AX signals (**Supplementary Figure 1G**, white triangle, panel 1), while the γH2AX intensity reduces as spermatocytes progress through the zygotene phase (**Supplementary Figure 1G**, white arrow, panel 2). Pachytene spermatocytes are characterized by a bright DDX4 staining and foci of γH2AX accumulation in the sex vesicles (**Supplementary Figure 1G**, white arrowhead, panel 3). While all germ cells were pre-meiotic prior to culture, at week 6, more than 50% of the germ cells had progressed to leptotene (**Figure 2J**, white triangle) or zygotene (**Figure 2J**, white arrow) spermatocytes (**Figures 3B,C**). However, pachytene spermatocytes were absent. These findings suggest that meiotic progression was arrested at the zygotene phase in TOs in both strains. Importantly, the fraction of germ cells was significantly lower at week 6 in comparison to the initiation of the culture (C57BL/6J: $p = 0.0058$; CBAB6F1: $p = 0.0494$) (**Figure 3A**).

1LS-TOs Support Steroidogenic Activity

The steroidogenic activity of Leydig cells in the TOs was quantified by measuring testosterone concentrations in the culture media throughout the culture period, and fluctuations over time were compared between the strains (**Figure 3D**). In C57BL/6J TOs, the testosterone concentration decreased significantly in week 2 ($p = 0.0042$) and 3 ($p = 0.0220$) compared to week 1, but recovered to initial values from the fourth week onwards. In CBAB6F1 TOs, the testosterone concentration reduced significantly at week 2 ($p = 0.0138$) and remained low until the end of the culture period (week 6). Aside from week 1 when C57BL/6J TOs produced significantly more testosterone ($p = 0.0071$), there was no significant difference in testosterone levels between the strains at other time points.

Chimeric TOs Can Be Formed in Both Cellink-RGD and Cellink 2LSs

Short-Term 2LS Cultured Chimeric TOs Display a Homogeneous Morphology

The potential to cultivate GS cells in TOs made up of primary testicular cells was tested. For this, testicular cells derived from prepubertal C57BL/6J mice were mixed at a ratio of 2:1 with GS cells expressing an ubiquitous EGFP reporter transgene (**Figure 4A**; **Supplementary Figures 2A–D**). Because cellular aggregates fused to form larger structures exhibiting core degeneration in 1LSs (**Figure 2D**), the focus was first placed on improving TO morphology using basal medium by adapting the scaffold design. This was evaluated during short-term culture of 1 week [**Figures 4A**(1)**–D**]. To immobilize the chimeric cell mixtures inside the macropores during self-reorganization, scaffolds consisting of either Cellink-RGD or regular Cellink and an additional layer of bioink were 3D printed [pore diameter of 531,25 ± 46,22 µm; **Figures 1B,D**, **4A**(1)]. Both regular Cellink (**Figures 4B–D**) and Cellink-RGD 2LSs (**Supplementary Figure 2E**) resulted in similar single spheroidal-shaped aggregates (454 ± 100,78 µm in diameter) after 1 week of culture. However, while the aggregates floated freely in Cellink 2LSs (**Figures 4B,C**), they adhered to Cellink-RGD 2LSs (**Supplementary Figure 2E**). Nevertheless, the type of hydrogel did not have noticeable effects on the histology, as all samples showed formation of cord-like structures (**Figures 4B,C**; **Supplementary Figure 2E**). In addition, EGFP-GS cells positive for DDX4 could be observed in the center and periphery of the chimeric aggregates (**Figure 4D**, white arrows). Noteworthy, dying cells were detected, but only in the periphery of the developing organoids (**Figure 4G**).

Long-Term 2LS Cultured Chimeric TOs Have a Compartmentalized Testicular Architecture and Consistent Tissue Health With Temporary Integration of GS Cells

Because the type of hydrogel did not affect histology during short-term culture, in the next experiment, the chimeric cell

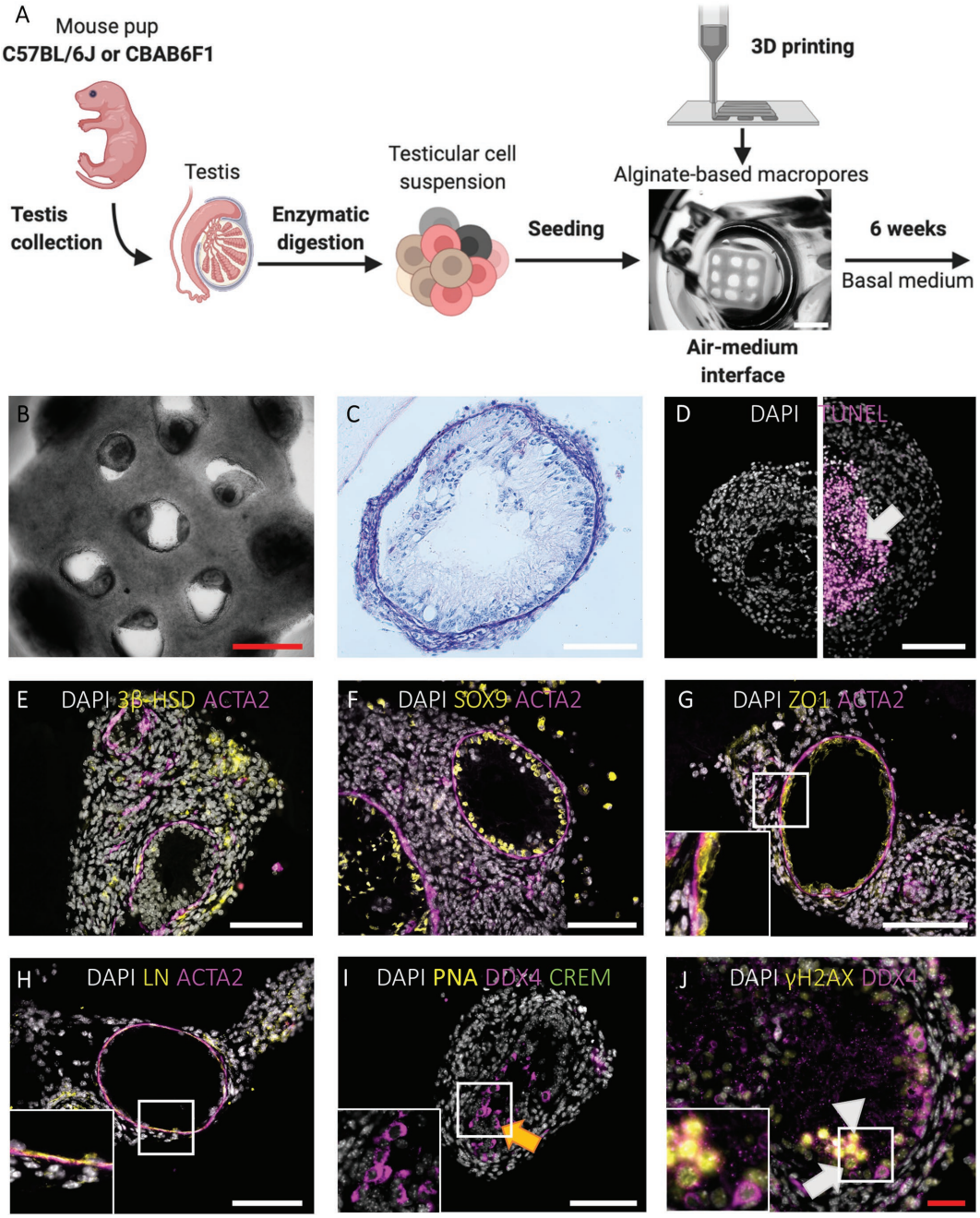

FIGURE 2 | Primary TOs in 1LS of both mouse strains restore in vivo testicular histology and support long-term survival and meiotic entry of early germ cells but display heterogeneous morphology. **(A)** Schematic representation of experimental procedure. Bar = 2 mm. **(B-C)** Gross morphological analysis of constructed TOs by bright field appearance **(B)** and PAS/Hematoxylin staining **(C)**. Bars = 500 µm (red) and 100 µm (white). **(D)** TUNEL assay revealed cell death in the central tissue region when early aggregates overgrew the macropores of 1LS to fuse into larger TOs (arrow). Bar = 100 µm. **(E-H)** Immunofluorescent staining of SSC niche cells and extracellular matrix: Leydig cells (3ß-HSD, **E**), Sertoli cells (SOX9, **F**), blood-testis-barrier (ZO1, **G**), laminin (LN, **H**), and peritubular myoid cells (ACTA2, **E-H**) are shown. Inserts correspond to boxed areas **(G-H)**. Bars = 100 µm. **(I,J)** Immunofluorescent staining of TOs for the constitutive germ cell marker DDX4 **(I-J)**, the post-meiotic germ cell markers CREM and PNA **(I)** and the meiotic marker γH2AX **(J)**. Orange arrow represents presumptive spermatocytes **(I)**. Boxed area includes leptotene (white triangle) and zygotene spermatocytes (white arrow) **(J)**. Bars = 100 µm (white) and 50 µm (red).

mixtures were cultured for 6 weeks in 2LSs composed of regular Cellink in basal medium with or without retinol supplementation in an attempt to overcome the meiotic block observed in the primary TOs in 1LSs [**Figure 4A**(2)]. Unlike primary TOs in 1LSs, early aggregates in 2LSs did not outgrow the pores to form larger TOs, but instead developed into uniform

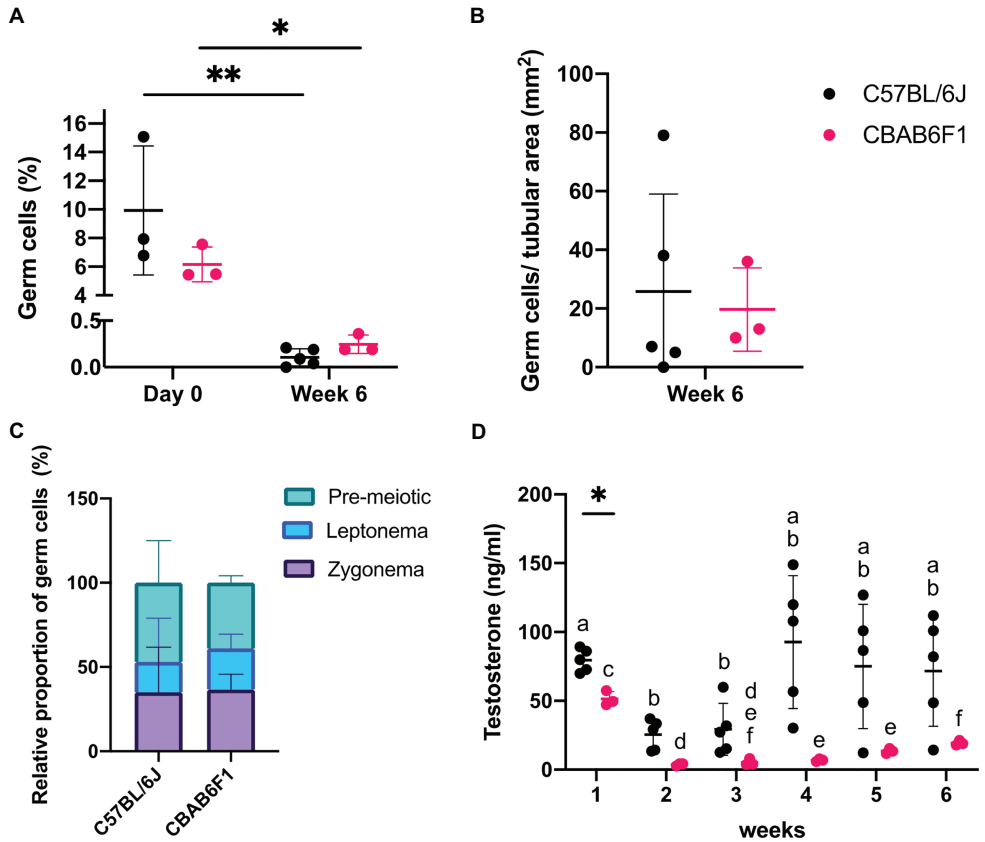

FIGURE 3 | Changing germ cell dynamics and detection of steroidogenic activity in primary 1LS-TOs. **(A)** Effect of time-course and mouse strain (C57BL/6J: $n = 5$, CBAB6F1: $n = 3$) on relative percentages of germ cell numbers. Asterisks show statistical significance. **(B)** Absolute germ cell numbers per mm^2 in primary TOs in 1LS at week 6. **(C)** Relative proportion of pre-meiotic and meiotic leptonema and zygonema in primary TOs in 1LS at day 0 and week 6 of culture. **(D)** Comparison of testosterone secretion in primary TOs between mouse strains at each timepoint. Different superscripts (a–f) show statistical significance over time within one mouse strain. Asterisk shows statistical significance between mouse strains on a specific time point.

spheroidal-shaped TOs measuring 416,36 ± 35,57 µm in diameter without core degeneration at week 6 (**Figures 4E–G**). Specifically, the chimeric TOs comprised two distinct compartments consisting of interstitial Leydig and peritubular myoid cells or tubular Sertoli cells (**Figures 4H–J**), separated by a tubular wall (**Figures 4E–F**). Nonetheless, germ cell differentiation was not observed, regardless of the culture medium; notably, DDX4 and EGFP$^+$ cells were completely absent at the end of the culture (**Supplementary Figure 2F**). Non-invasive follow-up of GS cells showed a dramatic decrease of EGFP+ cells over time (**Figures 4K–N**). A complete GS loss manifested in week 4 (**Figure 4M**).

DISCUSSION

In this study, 3D printed macropores served as a delimitation of the area in which testicular cells can grow in order to optimize TO morphology and histology. The resulting TOs had compartmentalized testicular cells that reorganized to a seminiferous tubule-like structure with surrounding interstitium, representing the functional unit of the testes. These functionalized TOs supported survival of primary germ cells, but not GS cells, for at least 6 weeks along with germ cell differentiation up to the level of early meiosis. Long-term germ cell survival in compartmentalized TOs is already a big step toward achieving complete IVS.

These findings extend our previous work in which lower densities of prepubertal testicular cells from C57BL/6N$^{ACR\text{-}EGFP}$ mice formed spheroidal-shaped aggregates in the macropores of 3D printed Cellink-RGD scaffolds without recognizable testicular architecture (Baert et al., 2019). Here, we showed that increased testicular cell densities promoted tubulogenesis, resulting in structurally compartmentalized organoids. The fact that low cell densities were also found to affect organoid morphogenesis in pig, macaque, and human suggests that optimizing this parameter ensures proper TO formation (Sakib et al., 2019). In terms of histology, our 3D printed culture system revised existing TO cultures relying on self-assembly in ECM-free conditions. Complementary to Edmonds and Woodruff (2020) (394 µm in diameter), we also successfully generated small compartmentalized TOs containing only one tubule. Additionally, we extended the germ cell survival to 6 weeks, likely due to cultivation at the air-medium interface. Simultaneous access to air and nutrients

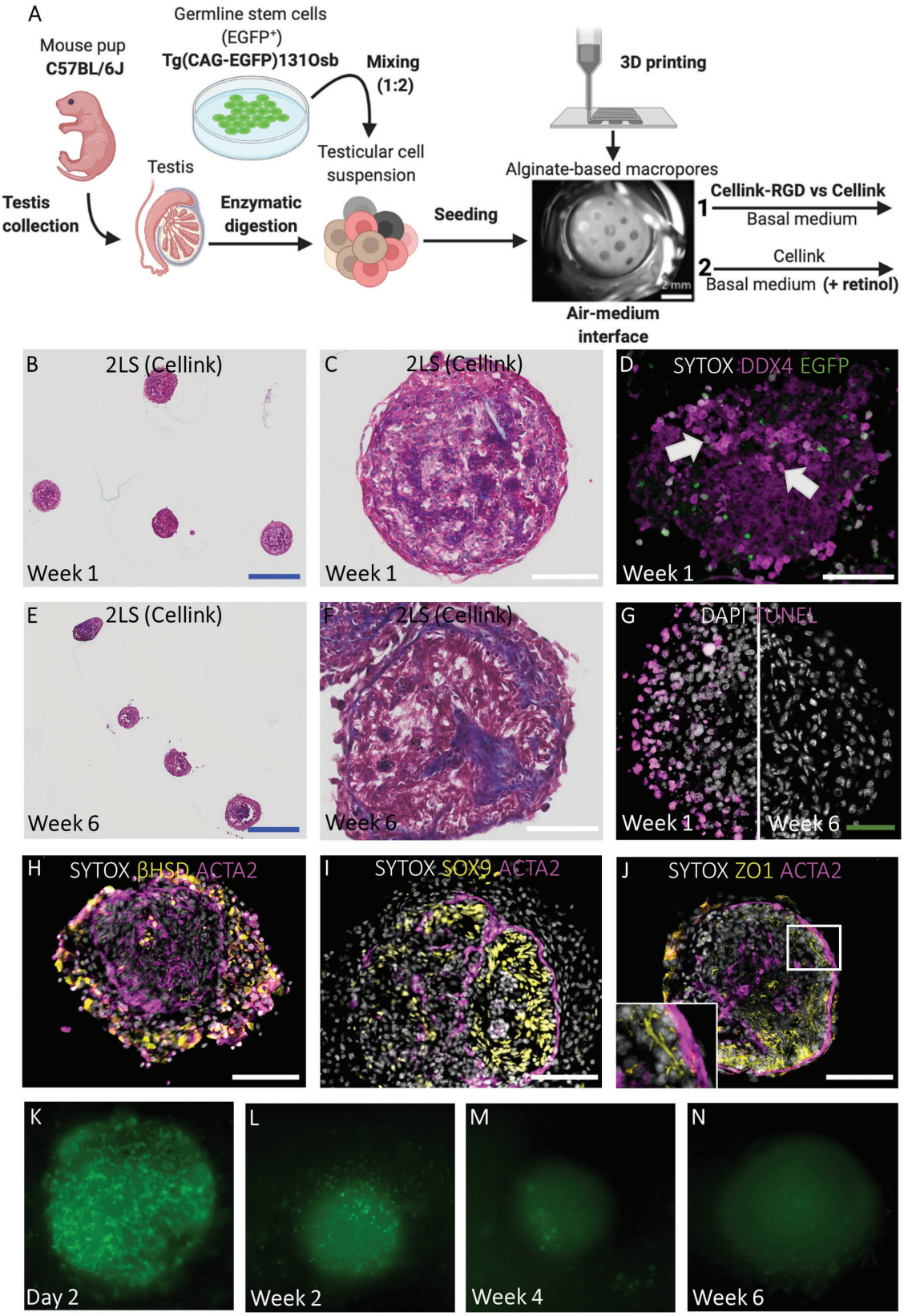

FIGURE 4 | Chimeric TOs in regular 2LSs display uniform morphology and restore components of the SSC niche but show germ cell loss. **(A)** Schematic representation of experimental procedure. First, modified Cellink-RGD and regular Cellink were compared for their ability to immobilize reorganizing chimeric cell

(Continued)

FIGURE 4 | mixtures inside the macropores of 2LS (1). Afterwards, basal medium was supplemented with retinol to promote spermatogenesis during long-term culture of TOs in 2LS composed of regular Cellink (2). Bar = 2 mm. **(B, C, E, F)** Histological examination by Masson's trichrome staining of chimeric TOs in Cellink 2LS after short-term **(B, C)** and long-term **(E, F)** culture. Bars = 500 µm (blue) and 100 µm (white). **(D)** Integration of DDX4+/EGFP+ cells (white arrows) into chimeric TOs after short-term culture. Bar = 100 µm. **(G)** Immunofluorescent TUNEL assay to assess cell death: death cells located at the periphery of 2LS-TOs the first week, but were absent at week 6. Bar = 50 µm. **(H-J)** Immunofluorescent stainings of SSC niche: Leydig cells (3ß-HSD, **H**), Sertoli cells (SOX9, **I**), blood-testis-barrier (ZO1, **J**) and peritubular myoid cells (ACTA2, **H-J**) are shown. Inset corresponds to boxed area **(J)**. Bar = 100 µm. **(K-N)** Non-invasive analysis of EGFP cells during long-term culture: day 2 **(K)**, week 2 **(L)**, week 4 **(M)**, week 6 **(N)**.

has been shown to be critical in organ cultures for germ cell functionality (Sato et al., 2011). Building on their organ culture expertise, Ogawa and colleagues generated millimeter-sized TOs on agarose pillars with, similar to our observations, meiotic cell formation during long-term cultures (Yokonishi et al., 2013). However, the large organoid size may have limited the diffusion of survival factors, resulting in signs of degeneration in the central part of their TOs. It was previously suggested that the integrity of testicular tissue fragments is optimal when they have a diameter of 300–400 µm for proper exchange of O_2 and nutrients (Komeya et al., 2016). Yet, in the current study, pore outgrowth in 1LSs and fusion of adjacent aggregates resulted in large TOs with uneven morphology and structure, displaying central degeneration and possibly variable testosterone production as well. The switch to 2LS and non-cell-interactive Cellink bioink made of inert alginate-nanocellulose further promoted cell entrapment and, thus, intercellular interactions. The resulting chimeric TOs measured approximatively 416 µm in diameter and did not display central degeneration. Considering uniform TOs hold more consistent readouts, their formation by means of adaptable 3D printed scaffolds would greatly help in developing a robust culture system for basic science and applications in reproductive toxicity testing, drug discovery and fertility preservation.

We generated TOs using cells from the C57BL/6J and the CBAB6F1 strains, both being popular models for *in vitro* gametogenesis research (Portela et al., 2019; Akin et al., 2021). Although tubule-like structures carrying germ cells were formed in TOs after 6 weeks of culture, germ cells from both strains did not progress through meiosis. This was unexpected since the same culture medium resulted in spermatogenesis in murine organ culture (Reda et al., 2017) and in cellular aggregates from transgenic C57BL/6N$^{ACR\text{-}GFP}$ mice in our previous 3D printing model (Baert et al., 2019). Of note, the location of the transgene in the genome of C57BL/6N$^{ACR\text{-}GFP}$ mice is unknown (Hasuwa et al., 2010). Also, Substrain-dependent differences exist (Jankovicova et al., 2016). Their influence on IVS has never been studied before and, consequently, cannot be ruled out. Moreover, TOs, which are reassembled testicular cell suspensions, may require an adapted version of the medium used in the organ culture system.

For instance, to determine whether the lack of complete spermatogenesis in our TOs could also in part be attributed to a failure to accurately represent a physiologically relevant testis surrogate, somatic support and Leydig cell function were assessed. Here, we demonstrated the development of a tubular lumen, as well as junctional specializations by Sertoli cells, both of which are signs of post-pubertal maturity. Concerning Leydig cell functionality, testosterone from C57BL/6J and CBAB6F1 primary TOs showed an unexpected drop after 1 week of culture, which corresponds with the start of puberty in mice at 10 days post-partum (Michael et al., 1980). In C57BL/6 male mice, testosterone was shown to rise from puberty and peaks at 40 days (Bell, 2018). Similar peaks of testosterone were absent in the TO cultures, regardless of the strain. The discrepancy in testosterone secretion patterns between the TOs and *in vivo* levels could (in part) explain the observed meiotic block. Even though it is not necessary in organ cultures to achieve IVS (Sato et al., 2011), in future TO experiments, the steroidogenic activity of Leydig cells could be stimulated by gonadotrophins from day 12 onwards to obtain strong testosterone levels as previously shown (Edmonds and Woodruff, 2020).

Additionally, while tissue handling has been reported to disturb spermatogenesis in organ culture *via* activation of the innate immunity (Abe et al., 2020), the successive processes of freezing-thawing and enzymatic digestion of the mouse testes to form TOs possibly afflicted even greater insult and may need to be countered.

Finally, even though TOs showed somatic cell reorganization, the relatively low abundance of germ cells reveals an inherent disturbance and requires particular attention as spermatogonia are essential determinants of sperm output. In contrast to testicular organ culture in which the architecture is preserved, the absence of a niche for the SSCs during the first days of TO culture may have triggered germ cell apoptosis. Previously, germ cells left without somatic support in aggregates have been reported to be vulnerable *in vitro* (Reda et al., 2014). This further indicates that the culture media will have to be optimized to enhance germ cell maintenance during the first weeks of cell reorganization. In addition to assessing the ability of TOs to support primary germ cells, we also investigated the possibility to grow and differentiate transgenic EGFP$^+$ GS cells in chimeric TOs. For this, retinol supplementation was tested as vitamin A metabolites play an essential role in SSC and GS cell differentiation (Arkoun et al., 2015; Wang et al., 2016). Strikingly, the amount of EGFP$^+$ germ cells decreased drastically during the first weeks to finally disappear at week 4, hindering the evaluation of retinol supplementation on GS cell differentiation. Compared to our study, EGFP signals lasted longer in the reconstructed tubule-like structures of Ogawa's group, reaching maxima in weeks 4–8 (Yokonishi et al., 2013). The addition of glial cell line-derived neurotrophic factor during reassembly may have benefited germ cell maintenance because of its known positive effects on SSC self-renewal (Kanatsu-Shinohara et al., 2003). Also, the possibility exists that unfavorable cell ratios could have resulted in inadequate support of germ cells from the somatic cells following the incorporation of GS cells.

To conclude, we reported the formation of TOs with compartmentalized architecture, supporting long-term survival and meiotic entry of early germ cells. Albeit this progress, further optimization of the culture medium will be necessary before full spermatogenesis can be obtained from primary germ cells and GS cells in the described TOs. The opportunity to manipulate multiple cell types in TO cultures will accelerate our understanding of testis development and function, but also enable generation of testicular disease and cancer models (Alves-Lopes and Stukenborg, 2018). In that regard, GS cells can be cultured over several years and are in combinations with TOs a valuable tool for studies on gene functions in spermatogenesis (Kanatsu-Shinohara, 2005; Takehashi et al., 2007). Taken further, germline genome editing in TO cultures may one day permit patients suffering from a genetic condition to father healthy children with healthy sperm. Finally, the use of TOs as a drug screening model will reduce the number of animals for the high demanding reproductive toxicity studies (Rovida, 2009).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee for the use of Laboratory Animals of the Vrije Universiteit Brussel Monash Medical Centre B Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

GR, RH, KL, EG, and YB designed the study. GR and YB performed the experiments, the analyses, and interpretation of the data. YB and EG obtained funding. GR wrote the manuscript. YB supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.757565/full#supplementary-material

Supplementary Figure 1 | Control immunofluorescent stainings of murine adult testicular tissue. **(A)** Immunofluorescence analysis of cell death (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, TUNEL, **A**) and the somatic testicular cells **(B–E)** and extracellular matrix (ECM; **C**) of the SSC niche: Leydig cells (ß-HSD, **B**), laminin (LN, **C**), Sertoli cells (SOX9, **D**), blood-testis-barrier (ZO1, **E**), and peritubular myoid cells (ACTA2, **B–E**) are shown. **(D,E)** Inset corresponds to boxed area. Bar = 100 μm. **(F,G)** Immunofluorescent stainings of the germ cell sub-types using the constitutive germ cell marker DDX4 **(F,G)** and the post-meiotic germ cell markers CREM **(F)** and PNA **(F)** or the meiotic marker γH2AX **(G)** to identify spermatocytes (**F**, red arrowhead), round spermatids (**F**, red arrow), elongated spermatids (**F**, red triangle), leptotene spermatocytes (**G**, white triangle, panel 1), zygotene spermatocytes (**G**, white arrow, panel 2), and pachytene spermatocytes (**G**, white arrowhead, panel 3). Bar = 100 μm (white).

Supplementary Figure 2 | Characterization germline stem (GS) cell and two-layer scaffold (2LS) cultures. **(A,B)** GS colonies on a MEF feeder exhibiting EGFP fluorescence **(B)**. **(C)** Flow cytometric analysis of EGFP expression in the GS cell culture before mixing with primary testicular cells. **(D)** The EGFP+ cells of the GS cell culture showed the distinct forward scatter/side scatter profile of GS cells. **(E)** Chimeric cell mixtures attached to Cellink-RGD macropores (dotted lines) during short-term culture. Bar = 100 μm. **(F)** DDX4+ and EGFP+ cells were absent at the end of the long-term culture in Cellink 2LS. Bar = 100 μm.

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Characterization and Survival of Human Infant Testicular Cells After Direct Xenotransplantation

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Background: Cryopreservation of prepubertal testicular tissue preserves spermatogonial stem cells (SSCs) that may be used to restore fertility in men at risk of infertility due to gonadotoxic treatments for either a malignant or non-malignant disease. Spermatogonial stem cell-based transplantation is a promising fertility restoration technique. Previously, we performed xenotransplantation of propagated SSCs from prepubertal testis and found human SSCs colonies within the recipient testes six weeks post-transplantation. In order to avoid the propagation step of SSCs *in vitro* that may cause genetic and epigenetic changes, we performed direct injection of single cell suspension in this study, which potentially may be safer and easier to be applied in future clinical applications.

Methods: Testis biopsies were obtained from 11 infant boys (median age 1.3 years, range 0.5-3.5) with cryptorchidism. Following enzymatic digestion, dissociated single-cell suspensions were prelabeled with green fluorescent dye and directly transplanted into seminiferous tubules of busulfan-treated mice. Six to nine weeks post-transplantation, the presence of gonocytes and SSCs was determined by whole-mount immunofluorescence for a number of germ cell markers (MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28), somatic cell markers (SOX9, CYP17A1).

Results: Following xenotransplantation human infant germ cells, consisting of gonocytes and SSCs, were shown to settle on the basal membrane of the recipient seminiferous tubules and form SSC colonies with expression of MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28. The colonization efficiency was approximately 6%. No human Sertoli cells were detected in the recipient mouse testes.

Conclusion: Xenotransplantation, without *in vitro* propagation, of testicular cell suspensions from infant boys with cryptorchidism resulted in colonization of mouse seminiferous tubules six to nine weeks post-transplantation. Spermatogonial stem cell-

based transplantation could be a therapeutic treatment for infertility of prepubertal boys with cryptorchidism and boys diagnosed with cancer. However, more studies are required to investigate whether the low number of the transplanted SSC is sufficient to secure the presence of sperm in the ejaculate of those patients over time.

Keywords: spermatogonial stem cell, gonocyte, spermatogonia, transplantation, cryptorchid, immature testis, infertility

INTRODUCTION

During the last decades, improved diagnostics and cancer treatments of children have resulted in more long-term survivors (1). However, chemotherapy and irradiation can lead to subfertility and sterility, which have serious negative effects on the life quality of survivors (2, 3). In post pubertal boys and men, semen cryopreservation prior to gonadotoxic therapy is the standard method for fertility preservation. However, this is not an option for prepubertal boys who are unable to provide a semen sample (4). Currently, cryopreservation of testicular tissue before gonadotoxic treatment, malignant and non-malignant diseases, is the only clinical method available to potentially preserve the fertility for prepubertal boys (5–7). Prepubertal testicular tissue cryopreservation (TTC) is an experimental method that preserves spermatogonial stem cells (SSCs), which are sperm progenitors that can potentially be used to restore spermatogenesis and ultimately produce spermatozoa in adulthood (8). Further, prepubertal boys treated for bilateral cryptorchidism may experience reduced sperm concentrations in adulthood, although the risks are attenuated by early orchidopexy (9). Nonetheless, around 17% to 25% of men with azoospermia have a history of cryptorchidism showing that cryptorchidism compromises fertility outcome, and in early childhood TTC performed at orchidopexy may be considered for more severe cases (10–12).

Spermatogonial stem cells appear at 3 to 12 months of age in boys usually in connection with the mini-puberty, and develop from gonocytes, which are mostly present during fetal and early neonatal life (13–15). Spermatogonial stem cells consist of A_{dark} (AdS) and A_{pale} spermatogonia. A_{dark} spermatogonia are phenotypically defined as spherical or ovoid cells with dark, dense chromatin in the nuclei frequently with a rarefaction zone, and are considered as the SSC reservoir (16). Physiologically, SSCs are thought to maintain the balance of self-renewal and differentiation to preserve the SSC pool and sustain perpetual spermatogenesis in adults. However, SSCs are a rare cell population in testis when considering "functional" capacity (17).

Transplantation of SSCs is considered a promising strategy to restore spermatogenesis and fertility in humans as proof of concept has been successfully demonstrated in rodents, domestic animals, and non-human primates (18). However, SSC transplantation in humans has not yet been successful (19). Optimal culture conditions for *in vitro* propagation of human SSCs are still lacking (20). Un-physiological *in vitro* conditions, such as enzymatic digestion, growth factors, and cytokines may result in oxidative stress and DNA damage (21, 22). In addition, zoonotic agents, including fetal bovine serum, also increase the risks for xenogeneic infections (23). Taken together, technical difficulties have hampered the translation from bench to clinic because of the lingering safety and ethical considerations. Although propagation of SSCs has been reported from infant boys under xeno-free conditions (24), the genetic and epigenetic stability of the SSCs still need to be determined before the application in clinical trials. Furthermore, the regulation for genetic and epigenetic determination also remains to be standardized.

Interestingly, in connection with transplantation of germ cells, both PGCs and gonocytes have, in addition to SSCs, shown the capability to support spermatogenesis in adult mammalian hosts post-transplantation (25, 26). Therefore, PGCs, gonocytes, and SSCs all possess the true stem cell potential and are referred to as male germline stem cells (27). Testis tissues, particularly from infant boys, contain gonocytes and SSCs, which may be used as a source of germline stem cells to restore spermatogenesis and produce sperm. Transplantation of neonatal or prepubertal germ cells from rodents has shown the capacity to colonize the host testis post-transplantation (28). However, it is not known whether human infant germ cells without *in vitro* propagation possess the capacity to colonize the testicular tubules post-transplantation.

In this study, we directly transplanted human infant testicular cells including gonocytes and SSCs into seminiferous tubules of sterilized nude mice to explore the feasibility of these stem cells to form colonies.

MATERIALS AND METHODS

Human Testis Tissue

Testis biopsies were obtained from 11 infant boys (median age 1.3 years, range 0.5-3.5) who underwent orchidopexy at the Department of Pediatric Surgery, Copenhagen University Hospital, Rigshospitalet (**Table 1**). Ten boys were diagnosed with congenital bilateral cryptorchidism, one with congenital unilateral cryptorchidism. None of the patients received hormonal therapy or were diagnosed with other conditions. The patients have previously been included in a study evaluating parental acceptance of experimental fertility preservation in young boys (29). Prior to tissue collection, informed consent was obtained from the parents of the patients for participating in the fertility preservation program and for donating a small testicular biopsy for research purpose. Consequently, the testicular biopsy from each testis was divided

TABLE 1 | Clinical and experimental parameters of infant boys with cryptorchidism.

Patient ID	Birth weight (g)	Diagnosis	Age at orchidopexy(year)	Testis location*	Serum FSH (IU/L)	Serum LH (IU/L)	Serum inhibin B (pg/ml)
#1	3075	bilateral	0.9	abdominal	1.60	0.37	147
#2	4184	bilateral	0.7	inguinal	0.57	0.36	222
#3	4000	bilateral	1.6	supra-scrotal	0.84	0.09	72
#4	3720	bilateral	1.7	supra-scrotal	0.64	0.14	76
#5	3590	bilateral	2.5	supra-scrotal	2.5	0.38	44
#6	4272	bilateral	3.5	supra-scrotal	0.56	0.05	57
#7	3200	bilateral	1.2	supra-scrotal	0.16	0.28	143
#8	3524	bilateral	1.0	inguinal	0.89	0.05	77
#9	4110	bilateral	0.5	annulus	0.57	0.85	280
#10	4910	unilateral	1.3	annulus	1.46	0.35	41
#11	3200	bilateral	1.4	supra-scrotal	0.81	0.11	70

**For the bilateral undescended testes, testis location indicated the location of the testis biopsy used for research.*

into three fragments: one for clinical TTC, one for routine pathological assessment, and one for research. The testis biopsy for pathological assessment was similar to the biopsy for research. The mean weight of the 11 testicular fragments for research use was 4mg. These testicular fragments were cryopreserved according to a previous published method (30). In brief, the tissue was equilibrated in 1.5 M ethylene glycol, 0.1 M sucrose, 10 mg/ml HSA for 20 min followed by a slow freezing procedure and storage in liquid nitrogen (30).

Handling of Animals

Nude mice (Naval Medical Research Institute (NMRI)-NU, Charles River, Denmark) were housed in groups, fed pellets and water ad libitum, and kept under controlled 12-hour light/12-hour dark cycles at 20-22°C. At eight weeks of age, each testis of mice was injected with 80 µg busulfan (B2635, Sigma-Aldrich) to eliminate endogenous spermatogenesis. The busulfan was dissolved in dimethyl sulfoxide (DMSO) and delivered in a volume of 20µl through two different sites (24, 31). Xenotransplantation was performed 4-5 weeks after busulfan treatment. Both injection and xenotransplantation were performed under anesthesia using Zoletil (Virbac, France), xylazin (Scanvet, Denmark), and butorphanol (Zoetis, New Jersey). Post-operative analgesia was provided by use of buprenorphine (Reckitt Benckiser; England, UK) and carprofen (Norbrook, England, UK). Following xenotransplantation, mice were single-housed until euthanasia. Euthanasia was done by cervical dislocation.

Histology, Cell Counting, Cryopreservation

Stieve's fixative was used for fixation and the infant testis tissue was embedded in paraffin and cut into 2-µm sections. The sections were stained with hematoxylin and eosin (HE) and germ cell markers including podoplanin (D2-40), cluster of differentiation 99 (CD99), octamer-binding transcription factor (Oct3/4), placental alkaline phosphatase (PLAP), and KIT proto-oncogene (C-KIT) following the same protocol as previously described (32).

Spermatogonia stem cells with or without the presence of gonocytes, constituted the germ cells within the infant cryptorchid testes. The total number of germ cells that included both SSCs and gonocytes was counted. The measurements of the number of germ cells and A_{dark} spermatogonia per cross-sectional seminiferous tubules was performed as previously described (33, 34) in at least 100 and 250 cross-sectional tubules per testicular biopsy, respectively. These measurements were carried out in a blinded fashion as a prognostic effort for evaluating the fertility potential.

To estimate the number of germ cells before xenotransplantation, we analyzed the germ cells within the testicular biopsy used for pathological assessment (**Figure 2A**). All sections used for pathological assessment were visualized digitally using a NanoZoomer digital pathology scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and quantified (total number of germ cells/surface area) with NDP viewer software (Hamamatsu Photonics K.K.). The seminiferous tubules were examined under a magnification of 40 x, whereas the measurement of surface area (excluding tunica albuginea) was carried out using a magnification of 5 x. All digital measurements were carried out blinded by two investigators and the final estimation of the germ cell density was presented as the mean number of germ cells/mm^3 from six non-serial sections. The germ cell density was calculated according to the following formula:

$$D = \frac{N}{A(d-t)}$$

D, density; N, number of germ cell counted; A, area of tissue on section; d, diameter of germ cell; t, thickness of the section. A prerequisite of this formula is equal distribution of cells in the section. The mean diameter of germ cells was measured from 10 germ cells per patient and only germ cells with the nucleoli visible were included for measurement. Within our samples, the diameter of the germ cell was 14 ± 1 µm (mean ± SD). The number of human germ cells prepared for each recipient was: mean weight of 11 testicular biopsies multiplied by the germ cell density (D).

The biopsies for research were placed in McCoy 5A medium (modified 22330-021, Gibco, Life Technologies, Paisley, UK) immediately after surgery for transportation to the laboratory (10 min. transport) where TTC was performed (30). The testis biopsy used for counting derived from the same biopsy which was used for xenotransplantation.

Cell Isolation and Xenotransplantation

Frozen testicular biopsies were thawed by three steps for 10 min in each medium: 1) 0.75 M ethylene glycol, 0.25 M sucrose in PBS, and 10 mg/ml Human serum albumin (HSA) (CSL Behring, Germany); 2) 0.25 M sucrose in PBS and 10 mg/ml HSA; 3) PBS and 10 mg/ml HSA according to a previously published method (30). Immediately after thawing, testicular biopsies were digested in α-MEM media supplemented with 2 mg/ml Collagenase type I (Worthington), 2 mg/ml Hyaluronidase type II (Sigma), 2 mg/ml Trypsin TRL3 (Worthington), and 16 μg/ml DNase I (Sigma) for 15 min at 37°C. After centrifugation, digested tissues were resuspended in Collagenase type I, Hyaluronidase type II, and DNase I at conditions similar to the first digestion and incubated for 30 min at 37°C. Human serum albumin 10 mg/ml was used to quench the enzyme activity. The cell suspensions were filtered through a 70 μm and subsequently a 40 μm strainer. Before transplantation, suspended cells were prelabeled with a green fluorescent dye PKH-67 (Sigma) according to manufacturer instructions (35). At transplantation, 15 μl containing 10^5 of testicular cells and 0.04% trypan blue (Sigma) were injected into the seminiferous tubules of recipient testis through the efferent duct (**Figure 1**).

Immunostaining

At 6 to 9 weeks post-transplantation, recipient testes were harvested and cut into a large (4/5 of the intact testis) and a small piece (1/5 of the intact testis). The large piece was used for analyzing human testicular cell colonization by whole-mount immunofluorescence in a three-dimensional arrangement. The small piece was fixed in formalin, embedded in paraffin, and cut into 5-μm sections for immunohistochemical staining. Moreover, immunohistochemistry was used to identify human cells using anti-human nuclear antigen antibody (anti-H). For the whole-mount immunofluorescence staining, tunica

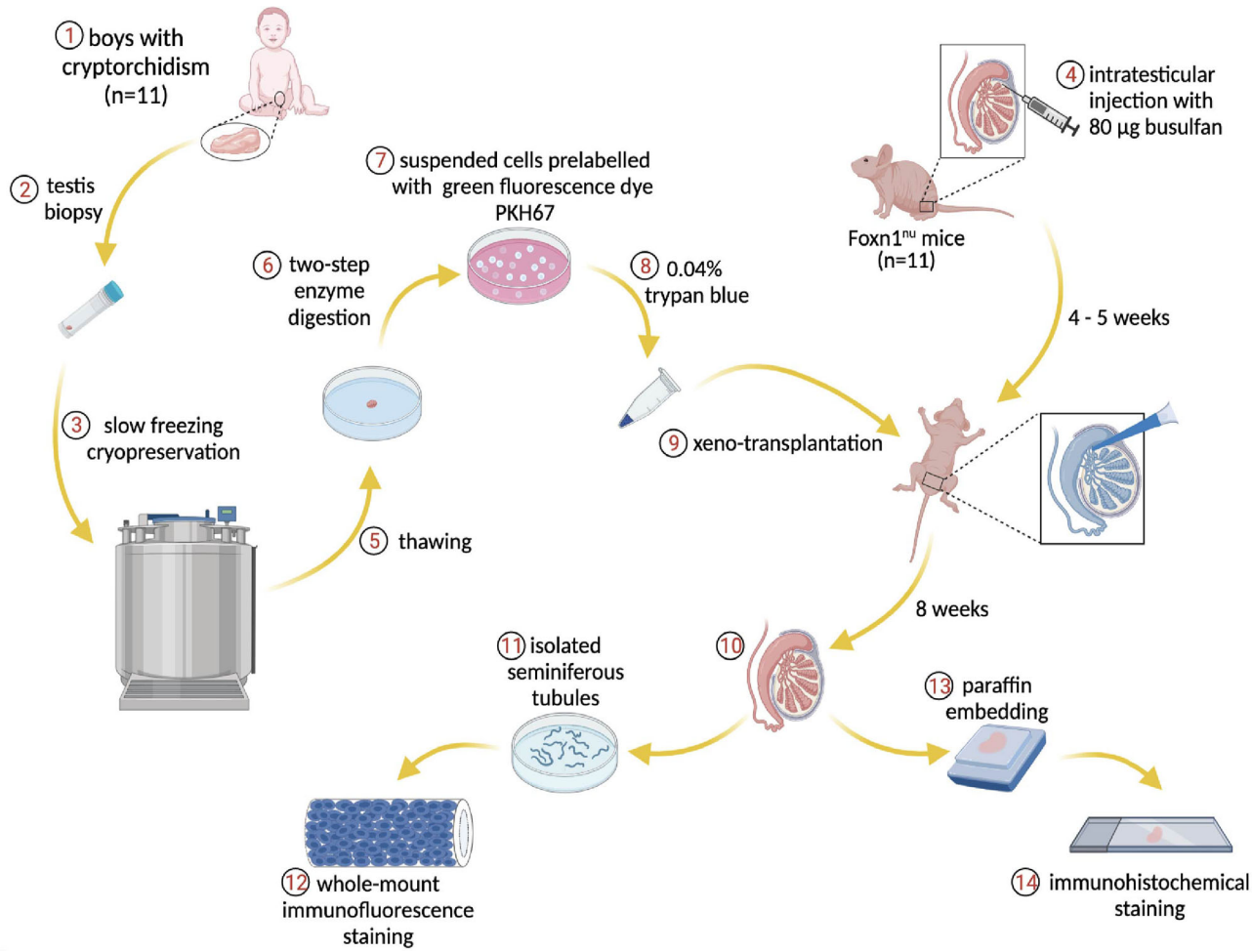

FIGURE 1 | Schematic overview of the experiment. Donor testis biopsies from infant boys with cryptorchidism underwent a frozen-thawed procedure and were dissociated through two-step enzymatic digestion. The suspended cells were prelabeled with PKH67 for tracing human cells within the recipient mouse seminiferous tubules post-transplantation. The endogenous spermatogenesis of recipient mice was eliminated by busulfan treatment. The human testicular cells were injected into the mouse tubules and the transplantation process was visible *via* trypan blue. Eight weeks later, the testes were harvested for immunostaining and further analysis.

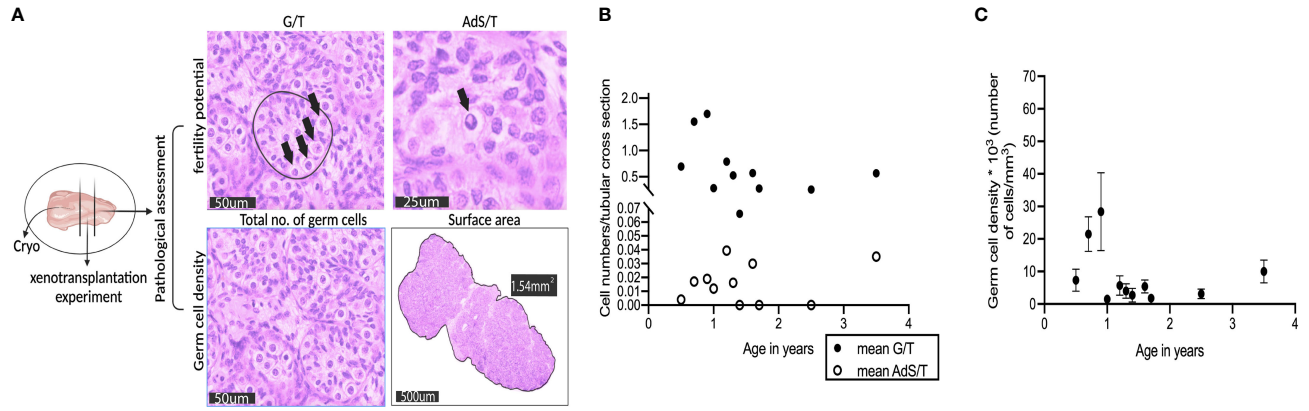

FIGURE 2 | Characterization of donor testis biopsies from infant boys with cryptorchidism. **(A)** The testis biopsies were cut into three parts. The bigger biopsy was cryopreserved in a biobank for fertility preservation, and the size of the other two pieces was similar, one for pathological assessment of the fertility potential and germ cell density, the other for experimental use. All four figures were from a testis biopsy from a 0.9-year-old infant boy with bilateral cryptorchidism (Patient ID #1). Germ cells per tubular cross section (G/T), A_{dark} spermatogonia per tubular cross section (AdS/T). G/T figure: four germ cells (black arrow) within one seminiferous tubule (black circle), scale bar, 50μm; AdS/T figure: an A_{dark} spermatogonium (black arrow), scale bar, 25 μm; total number of germ cells figure: scale bar, 50 μm; surface area figure: 1.54 mm^2, scale bar, 500 μm. **(B)** The number of germ cells (G/T) and A_{dark} spermatogonia (AdS/T) per tubular-cross section from 11 testis biopsies, x-axis represents age in years. **(C)** Germ cell density was the total number of germ cells per mm^3 on tubular sections from 11 testis biopsies, x-axis represents age in years. Dots represent the mean value; error bars represent standard deviation (SD).

albuginea of the recipient testis was removed, and the seminiferous tubules were gently separated and digested with DNase I (1 mg/ml) and Collagenase IV (1 mg/ml) in Hanks' balanced salt solution (HBSS). Dispersed tubules were fixed in 4% formaldehyde and washed in PBS. Tubules were incubated in 10% methanol and blocked with 2% bovine serum albumin and 0.5% Triton X-100 in PBS.

Histological sections were incubated overnight at 4°C with the following primary antibodies (**Supplementary Table 1**), anti-melanoma antigen genes-A mouse monoclonal antibody (MAGEA), anti-G antigen (GAGE) mouse monoclonal antibody. MAGEA and GAGE are cancer/testis-associated proteins encoded by gene clusters located on the X chromosome and expressed in spermatogonia and primary spermatocytes in adult testis and also in migrating primordial germ cells in human embryo (36–38). We also used anti-ubiquitin carboxyl-terminal hydrolase L1 mouse monoclonal antibody (UCHL1) which is a spermatogonial marker (20, 39); anti-Sal-like protein 4 mouse monoclonal antibody (SALL4) is a member of *sal*-gene family of transcription factors and a conserved marker of spermatogonia (40, 41); anti-undifferentiated embryonic cell transcriptional factor 1 mouse monoclonal antibody (UTF1) is a spermatogonial marker (42); and anti-LIN28 mouse monoclonal antibody (LIN28) is a RNA-binding protein expressed in gonocyte of fetal testis and spermatogonia of postnatal testis (43). Moreover, anti-SOX9 rabbit polyclonal antibody (SOX9) was used as a Sertoli cell marker, anti-cytochrome P450 17A1 goat polyclonal antibody (CYP17A1) was used as a Leydig cell marker. After wash in Tris-buffered saline with Tween20® (Sigma)(TBST), the slides were incubated with secondary antibodies anti-mouse Alexa 594/anti-rabbit Alexa 594/anti-goat Alexa 568 (1:500, Jackson ImmunoResearch) for 1 hour at room temperature (RT). After nuclear staining with 4',6 - diamidino-2-phenylindole (DAPI), the seminiferous tubules were mounted on slides with antifade reagent (Invitrogen). The green cells (human cells prelabeled with PKH67) were counted and cells at least 150μm apart were considered as different colonies. The colonization efficiency was calculated as no. human germ cell colonies identified/no. human germ cells injected. The testes from recipients no.1 to no.8 were all harvested 8 weeks post-transplantation, whereas no.9, no.10, no.11 were collected at 7 weeks, 6 weeks, 9 weeks post-transplantation, respectively (**Table 2**).

For the immunohistochemical analysis, slides were deparaffinized and rehydrated with series of graded ethanol, followed by antigen retrieval in TEG buffer (10 mM Tris, 0.5 mM ethylene glycol-bis (2-aminoethylehter)-N, N, N', N'-tetraacetic acid (EGTA), pH 9.0) in boiling water for 30 min. Endogenous peroxidases were blocked by 0.5% H_2O_2 for 15 min, and non-specific binding was blocked with 4% bovine serum albumin (BSA) and 5% donkey serum (DS) for 30 min at RT. The slides were incubated at +4°C overnight with primary antibodies, MAGEA/GAGE/UCHL1/SALL4/UTF1/LIN28/Sox9/CYP17A1 and anti-H (**Supplementary Table 1**). After wash in TBST, the secondary antibody was added, donkey anti-mouse/rabbit/goat antibody (Dako) horseradish peroxidase for 30 min at RT, visualized with 3,3'-diaminobenzidine tetrahydrochloride (Dako) for 1-2 min, counterstained with Mayer's hematoxylin and mounted with Pertex (Histolab). All the slides were analyzed, and images were taken under a microscope with a digital camera (Leica).

Statistical Analysis

Individual values were shown as mean ± standard deviation (SD). GraphPad Prism version 8.0 was used for statistical analyses. The data of colonization efficiency, hormone levels, and age followed a normal distribution. Correlations between

TABLE 2 | Colonization efficiency of germ cells from infant boys with cryptorchidism.

Recipient	Testis	Patient ID	No. human germ cells prepared[a]	% human cells injected to recipient[a]	No. human germ cells actually injected[c]	No. human germ cell colonies found after transplantation[b]	No. human germ cells found after transplantation[b]	% mean colonization efficiency[c]
1	left	#1	56798	5 - 15	2840 - 8520	43	256	1.1
	right			5 - 15	2840 - 8520	51	303	
2	left	#2	43097	5 - 15	2155 - 6465	21	128	0.6
	right			5 - 15	2155 - 6465	16	96	
3	left	#3	10703	10 - 20	1070 - 2141	19	112	6.3
	right			5 - 15	535 - 1605	90	542	
4	left	#4	3681	15 - 25	552 - 920	19	112	3.8
	right			25 - 35	920 - 1288	51	303	
5	left	#5	6128	20 -30	1226 - 1838	85	511	9.0
	right			5 - 15	306 - 919	56	335	
6	left	#6	19841	5 - 15	992 - 2976	77	463	3.0
	right		19841	100	19841	149	894	
7	left	#7	11303	10 - 20	1130 - 2261	85	693	3.1
	right			60 - 70	6782 - 7912	37	187	
8	left	#8	3025	5 - 15	151 - 454	75	702	18.6
	right			30 - 40	908 - 1210	43	427	
9	left (7 weeks)	#9	14696	5 - 15	735 - 2204	10	96	0.5
	right (7weeks)			5 - 15	735 - 2204	0	0	
10	left (6 weeks)	#10	7959	30 - 40	2388 - 3184	98	1127	1.8
	right (6 weeks)			100	7959	1	10	
11	left (9 weeks)	#11	5361	60 - 70	3217 - 3753	86	942	6.4
	right (9 weeks)			10 - 20	536 - 1072	74	817	

Recipient testis no. 1 to no. 8 were harvested 8 weeks after xenotransplantation; no.9 to no.11 were harvested 7, 6, 9 weeks after xenotransplantation.
[a]estimated number; [b]counted number; [c]calculated number.
*No. human germ cells actually injected = No. human germ cells prepared * % human cells actually injected to recipient; Mean colonization efficiency % = (No. human germ cell colonies obtained after transplantation/No. human germ cells actually injected) * 100.*

colonization efficiency and hormone levels/age were tested by Pearson correlation coefficient. The multiple comparison was performed with Kruskal – Wallis test among colonization efficiency at different weeks. *P* values less than 0.05 were considered statistically significant.

RESULTS

Characterization of Infant Testes

Spermatogonia were located on the basal membrane, while the gonocytes were present in the center of the tubules. The number of germ cells per tubular cross-section (G/T) ranged from 0.07 to 1.70 among patients while some testis samples were lacking A_{dark} spermatogonia (**Figure 2B**). According to the formula of germ cell density, we found that the initial mean germ cell density (before xenotransplantation) was from 1513 to 28399 cells per mm^3 (**Figure 2C**). The weight of the testicular tissues before cryopreservation was 4 ± 3 mg. Combining the germ cell density and the weight of the testicular biopsies, we estimated the number of human germ cells prepared for each recipient (**Table 2**).

To evaluate the specificity of the immunohistochemical markers, reference testis tissues from two infant boys with cryptorchidism were used. MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28 were all expressed in germ cells located in the lumen and/or on the basal membrane of the seminiferous tubules. SOX9 was expressed in the nuclei of the Sertoli cells (**Figure 3**).

Colonization of Recipient Testes by Human Germ Cells

We found that human infant germ cells could form colonies within the recipient seminiferous tubules 8 weeks post-transplantation. Based on the appearance of the seminiferous tubules after histological staining, we were unable to identify human germ cells in meiosis (i.e., from preleptotene onwards). The actual location of human germ cells in the seminiferous tubules were on the basal membrane.

Whole-Mount Analysis of Germ Cell Colonization Efficiency

Whole-mount immunofluorescence staining identified human germ cells positive for both PKH67 and the germ cell markers, MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28 (**Figures 4**, **5**). By changing the focal plane of the microscope, we could observe these human germ cells on the outer layer of the tubules indicating that they were located on the basement membrane of

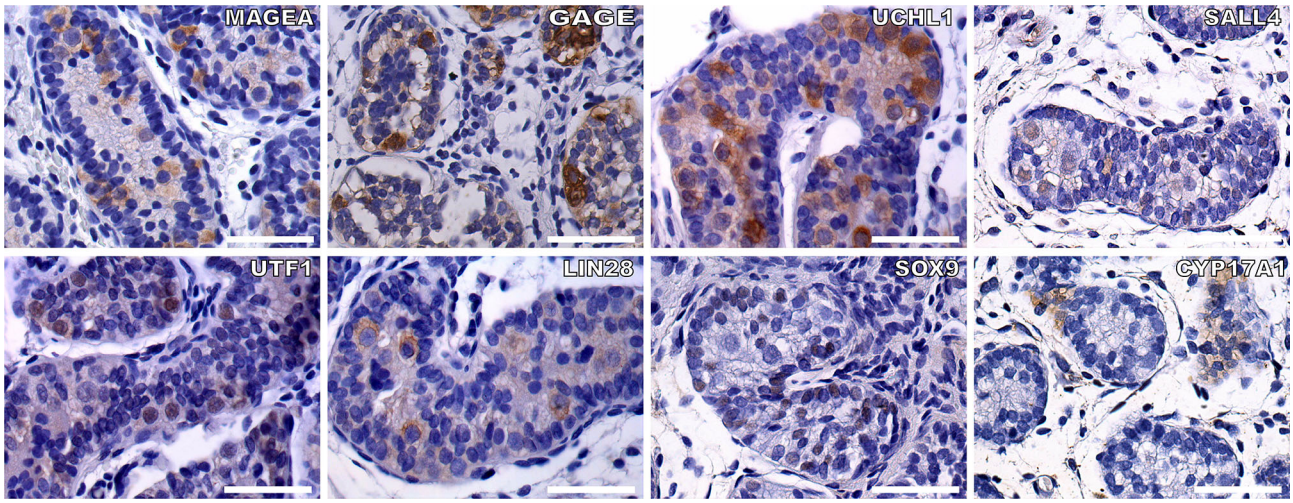

FIGURE 3 | To check the feasibility of the germ cell and somatic cell markers, we did IHC staining of germ cells and Sertoli cells within the seminiferous tubules from two infant boys with cryptorchidism. Germ cell markers included: MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28, the positive staining of germ cells within the seminiferous tubules (brown). Sertoli cells were indicated by SOX9 (brown). CYP17A1 stained the Leydig cells (brown). The nuclei were stained by hematoxylin (blue). Scale bar, 50 μm.

the tubules. The Sertoli cell marker SOX9 was not expressed in the nuclei of the green human cells, indicating that the colonies did not contain any Sertoli cells (**Figure 6**). We found one single human Leydig cell stained by CYP17A1 that survived in the recipient testis (**Supplementary Figure 1**). Thus, we considered the cells within the colonies were all germ cells. Human PKH67-positive cells stained green but not all germ cells were detected using each of the applied markers and showed that different phenotypes of human germ cells survived transplantation (**Figures 4**, **5**).

The mean colonization efficiency was 6.4% at nine weeks post-transplantation, and 1.8% and 5.7% at six weeks and eight weeks, respectively (**Table 2**), which was not significantly different. We injected the full volume of 15μl cell suspensions into the right side of recipient testis no.6 and no.10. However, the colonization efficiency (right side of recipient testis no.6) was only 1%. Almost all seminiferous tubules within the right-sided recipient testis of no. 10 became atrophic and solidified at six weeks post-transplantation and only 1 colony was found from the viable tubules. There was a positive correlation between the number of human germ cells actually injected and human germ cell colonies obtained eight weeks post-transplantation ($r = 0.50$, $P = 0.048$). (**Table 2**). No correlation was found between colonization efficiency and clinical parameters (serum hormones, age) (**Tables 1**, **2**).

Immunohistochemical Analysis

The anti-human nuclear antigen antibody expressed on the recipient testis sections also identified human cells and proved to survive human cells 8-weeks post-transplantation. The human cells were positioned on the basal membrane of the recipient seminiferous tubules (**Figure 7**).

DISCUSSION

This study demonstrated that germ cells from infant boys with cryptorchidism can colonize the recipient mouse testes and survive six to nine weeks post-transplantation without purification or propagation before transplantation. Our study showed that human germ cells were located in the niche on the basal membrane of the recipient seminiferous tubules and able to proliferate but failed to progress in sperm development. These results are in line with a previous report in which no spermatogenesis was detected after xenotransplantation human testicular cells for up to six months post-transplantation (19).

We showed that the survived human cells prelabeled with the dye PKH67 were SSCs with different phenotypes expressing MAGEA, GAGE, UCHL1, SALL4, UTF1, and LIN28. This confirms and extends a previous finding, that after transplantation of flow cytometry sorted human spermatogonia, different phenotypes of spermatogonia can result (41, 44). Thus, we confirmed that not only one human phenotype of infant SSCs has the capacity to colonize the recipient seminiferous tubules and settle in the niche. Therefore, it is likely that a variety of different phenotypes of SSCs may be used for transplantation in future clinical studies for fertility restoration in adult men.

Direct transplantation of infant germ cells without *in vitro* propagation has the advantages of reducing possible genetic and epigenetic changes due to culture conditions improving its safety and also, it is easier to be applied in a clinic in the future. A prerequisite for performing transplantation of only enzyme digested testis tissue is a sufficient number of transplanted germ cells settle in the proper niche to sustain renewed

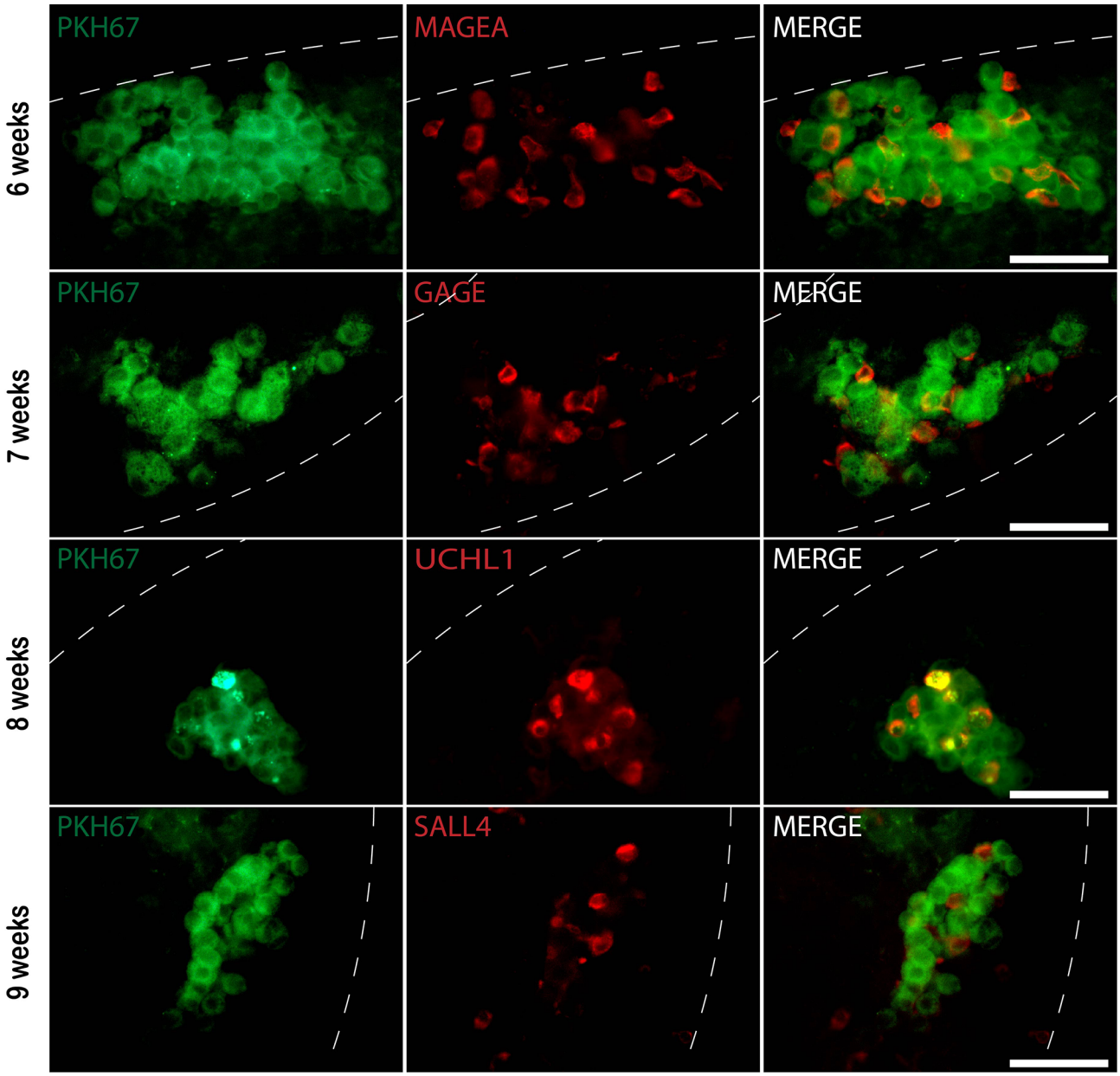

FIGURE 4 | Whole-mount immunofluorescence (IF) staining of recipient mouse seminiferous tubules xenotransplanted with donor human testicular cells prelabeled with PKH67. Six to nine weeks post-transplantation, PKH67 cells (green) were near the basement membrane of the mouse seminiferous tubules (white dotted lines) and co-stained with different germ cell markers (red). 6 weeks: Donor testis biopsy was from a 1.3-year-old boy (Patient ID #10). PKH67-positive cells (green) co-expressing germ cell marker MAGEA (red) within the recipient mouse tubule. 7, 8, and 9 weeks: Donor testis biopsies were from boys at 0.5, 2.5, 1.4 years old, respectively (Patient ID #9, #5, #11). PKH67-positive cells (green) were found inside the recipient tubules and expressed GAGE (red), UCHL1 (red), and SALL4 (red). The nuclei were stained by DAPI (blue). Scale bar, 50 μm.

spermatogenesis. Since data suggest that spontaneous spermatogenesis may occur after chemotherapy causing a severe depletion of SSCs (45), it may be hypothesized that only a few human SSCs, once in the proper microenvironment, may be sufficient for spermatogenesis to recover over time. Thus, after some chemotherapy regimen conditions, direct transplantation of even a small number of SSCs could result in sperm production during a long-term *in vivo* propagation of SSCs. Currently, it is unknown how fast spermatogenesis could potentially be re-established, but a considerable time may be required. However, even if some years were required for spermatogenesis to take place, this could still be achieved by transplanting applied to these boys as adolescents allowing them to be fertile as the normal age of fatherhood.

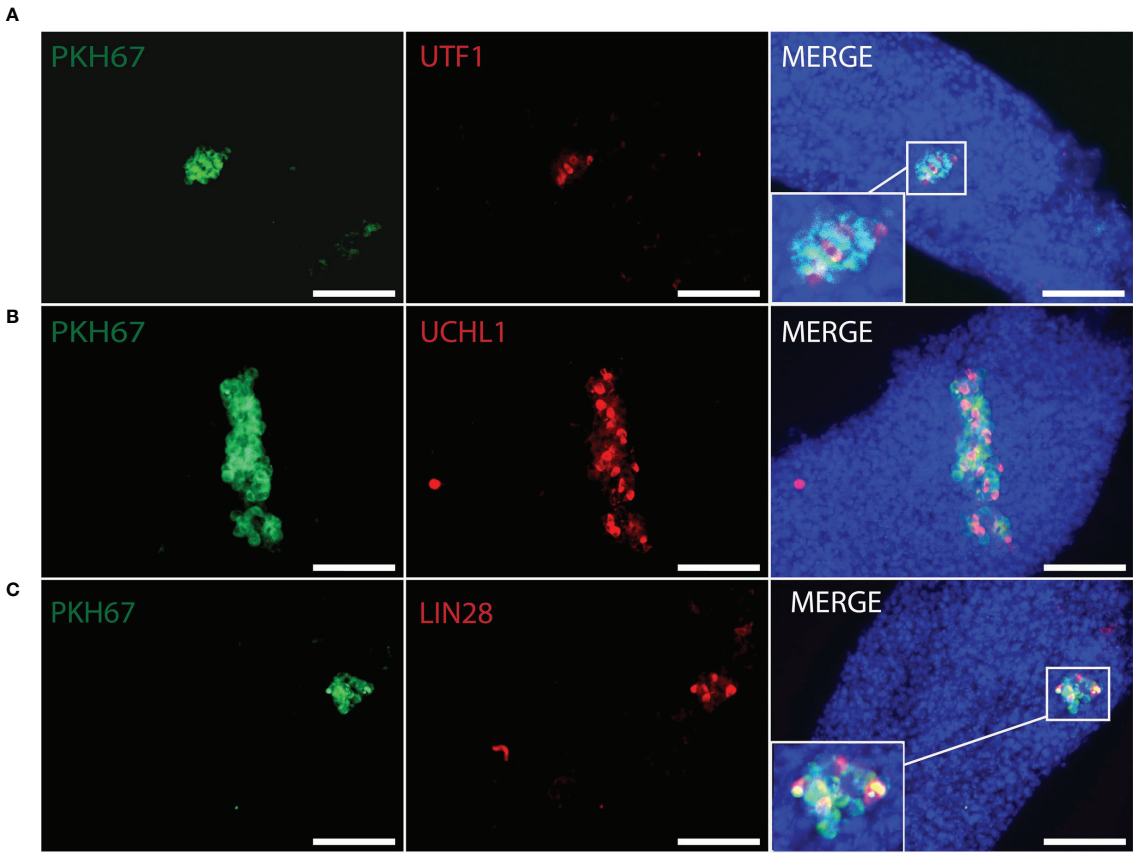

FIGURE 5 | Whole-mount detection of human germ cells xenotransplanted into recipient mouse seminiferous tubules by different germ cell markers. PKH67-positive cells (green) indicated human cells co-stained with **(A)** UTF1 (red), **(B)** UCHL1 (red), **(C)** LIN28 (red) within the recipient mouse tubules. Corresponding to figures **(A–C)**, donor testis biopsies were from Patient ID #11, #5, #5. DAPI (blue) for nuclear staining. Scale bar, 100 μm.

We were unable to detect human Sertoli cells at eight weeks following transplantation in the recipient testis. The absence of human Sertoli cells could be due to the fact that the recipient mouse Sertoli cells were not eliminated by the busulfan treatment, that was used to deprive endogenous germ cells to provide space for donor germ cells. Therefore, it is hypothesized that there was no extra space for the human Sertoli cells to settle down in the mouse tubules. Previously, studies have reported that after removing recipient endogenous Sertoli cells, donor Sertoli cells may colonize the recipient's seminiferous tubules (46, 47). Another explanation could be that the human Sertoli cells were phagocytized by the recipient Sertoli cells or macrophages. This is substantiated by results showing that donor germ cells which failed to attach to the basal membrane were phagocytized by the recipient Sertoli cells and that released sperm could be engulfed by macrophages (48). However, one study on transplantation of bovine testicular cells into mouse testes showed that some bovine Sertoli cells survived in mouse tubules two months post-transplantation (49). In our study, we did not stain all the tubules for somatic cell markers, and we cannot exclude that there could be a few donor somatic cells surviving in other parts of the testis. As we identified one single human Leydig cell that survived within mouse testis post-transplantation, further studies are needed to investigate whether human Sertoli cells and Leydig cells may survive in larger numbers in the recipient testis post-transplantation.

Human germ cells migrated to the basement membrane of the mouse seminiferous tubules, settled, and formed colonies. According to previous studies, one colony is often formed from just one SSC (50, 51). In our study, the number of human germ cell colonies formed after xenotransplantation was presumed to be equal to the number of survived human SSCs with proliferative activity. We found approximately 55 colonies per 10^5 testicular cells at eight weeks post-transplantation. Valli and colleagues reported four to nine colonies per 10^5 cells eight weeks post-transplantation. To enrich the colonization efficacy, they used fluorescence-activated cell sorting to sort out different phenotypes of SSCs followed by xenotransplantation and reported around 50 colonies per 10^5 cells within the recipient tubules two months post-transplantation (44). Thus, they achieved a 12-fold enrichment of colonies compared to the unsorted population (44). We reached colonization efficacy similar to the sorted

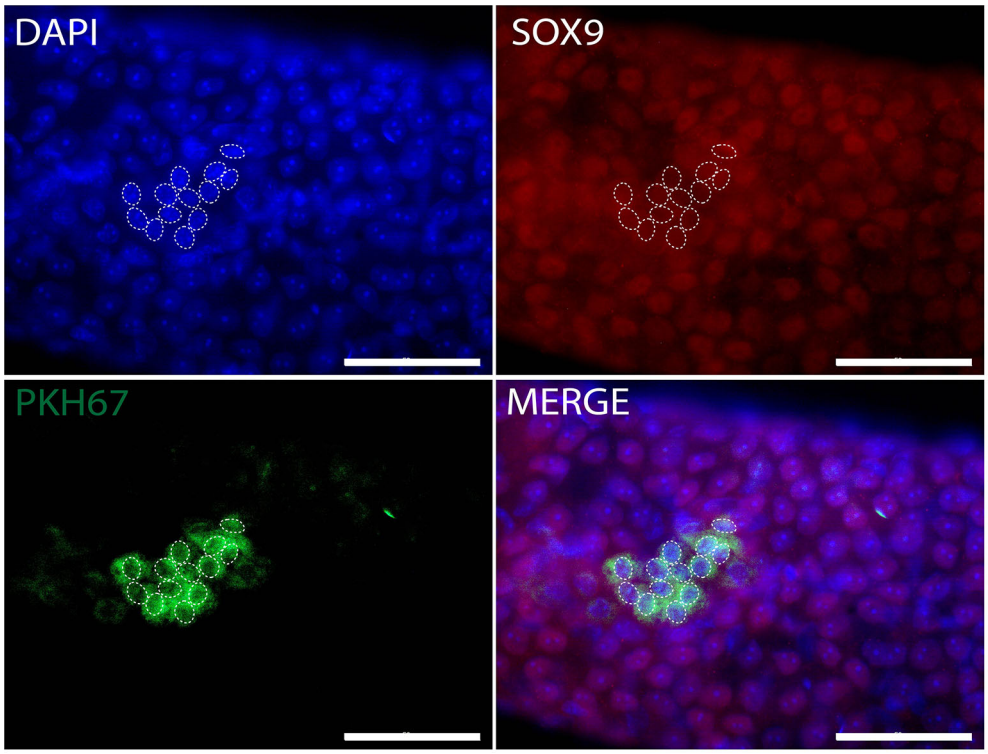

FIGURE 6 | Whole-mount detection of human Sertoli cells xenotransplanted into recipient mouse seminiferous tubules. PKH67-positive cells (green) indicating human cells without expression of Sertoli cell marker SOX9 (red). White dotted circles: PKH67-positive cells. The nuclei were stained by DAPI (blue). Scale bar, 50 μm.

fraction in their study. However, the colonization efficacy may be related to the age of the boys, which in our study was 0.5 – 3.5 years, while postpubertal donors (age 14 – 50 years) were used in Valli's study.

If the direct transplantation of a few human SSCs is not sufficient, then propagation of SSCs prior to transplantation is necessary. In order to evaluate the magnitude of SSC propagation required, Sadri et al. calculated that SSCs within a volume of 200ul of a prepubertal testis biopsy were required to be increased by 65-fold in order to colonize an adult normal-sized 13ml testis after SSCs transplantation (52, 53). According to the colonization efficiency of SSCs from animal auto-transplantation models, they assumed that the efficiency for human SSCs auto-transplantation is at least 5% and therefore a 1300-fold increase of human SSCs is required for a sufficient number of cells to justify transplantation. In our study, the volume of infant testis biopsy was approximately 4ul and the mean colonization efficiency was around 6% eight weeks post-transplantation. According to the calculation method from Sadri et al. (52), the number of SSCs within a 4ul biopsy from an infant testis is required to increase 3250-fold to contain sufficient cells to colonize an adult testis after transplantation. The concentration of SSCs differs between an infant and an adult testis and our results showed that infant testis biopsies had about 10-fold enrichment of SSC colonies compared to the biopsies from adult donors. Therefore, an approximate 5400-fold (3250-fold/10/6%) enrichment of SSCs would be necessary for sufficient repopulation of an adult testis. Thus, although these calculations are subject to several uncertainties, these results suggest that propagating SSCs *in vitro* is a necessary step.

In addition, colonization efficiency was crucial for obtaining sufficient SSC to repopulate the recipient testis. We found that patient no. 8 (age 1.0 year) where the least number of cells have been injected, the colonization efficiency was highest. In contrast, the lowest colonization efficiency was observed for patient no.2 and no.9 (age 0.7 and 0.5 years, respectively). These three patients were among the four youngest patients in our study. Therefore, our data do not support the age of the patient as a determining factor for colonization efficiency.

Successful spermatogenesis following transplantation of early-stage donor germ cells may also be related to the age of recipients. Mouse PGCs could produce spermatozoa in neonatal recipient mice (54), while macaque PGCs failed to achieve spermatogenesis in adult macaque recipients (25). For the future auto-transplantation of human SSCs from the infant or early prepubertal testis, the age of male recipients undergoing SSCs auto-transplantation could also be important.

It is a limitation of current study that the viability of different types of testicular cells immediately before xenotransplantation is not measured, including viability of germ cells (i.e., after cryopreservation, thawing, and enzymatic digestion), and this may affect the results obtained after xenotransplantation to the

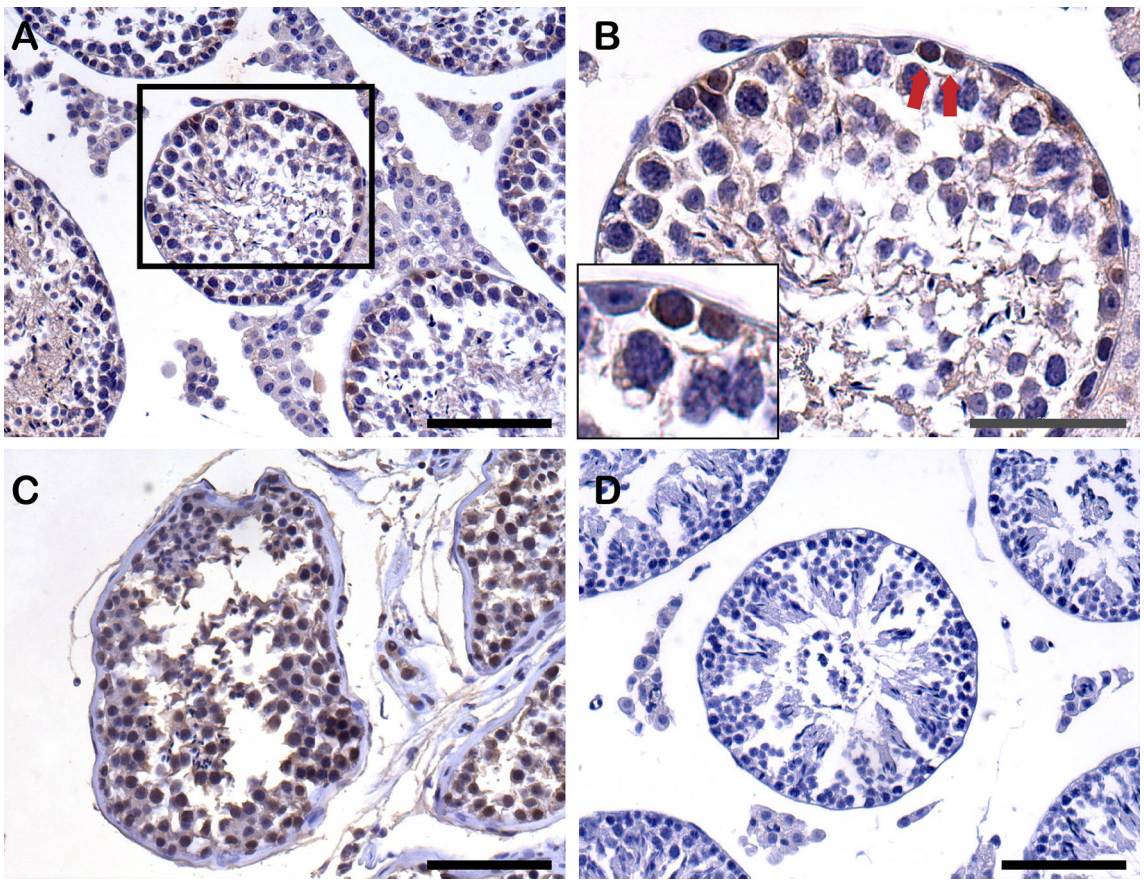

FIGURE 7 | Detection of human cells by anti-human nuclear antigen antibody (anti-H) by immunohistochemistry (IHC) staining on paraffin-embedded testis sections from recipients after xenotransplantation. **(A)** Human cells (anti-H positive cells) settled down on the basement membrane of recipient seminiferous tubules 8 weeks post-transplantation. Scale bar, 100 μm. **(B)** Higher magnification of recipient seminiferous tubule with red arrows indicating the anti-H positive human cells on the basement membrane. Scale bar, 50 μm. **(C)** Positive control of anti-H from normal human adult testis tissue. Scale bar, 100 μm. **(D)** Negative control of anti-H from mouse testis tissue. Scale bar, 100 μm.

recipient mice. However, we do not consider this to be a major factor influencing the results (partly based on own results).

In theory, the use of mouse monoclonal antibodies in mouse tissue is usually followed by difficulties in getting robust results. However, in this study the antibodies were used to detect human cells in IF, which in most instances were PKH67 positive cells. In addition, we checked that the anti-human nuclear antigen antibody (anti-H) showed no cross-reaction with mouse testicular cells (**Figure 7D**). Other studies have used a similar approach to ours (19, 55).

Collectively, our data suggest that human germ cells consisting of gonocytes and SSCs from infant boys with cryptorchidism are capable of colonizing mouse seminiferous tubules eight weeks post-transplantation. With the successful re-establishment of spermatogenesis following SSC auto-transplantation in rodents and non-human primates, it is expected that auto-transplantation of human germ cells will also result in sperm generation. Further studies to determine the minimum number of SSCs and the timeframe required for the initiation and establishment of spermatogenesis following SSC auto-transplantation are needed in order to evaluate the potential clinical application of direct SSC transplantation. However, it is possible that the number of donor SSCs needs to be enriched to improve chances for successful re-initiation of spermatogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethics Committee of Copenhagen (No. H-2 2012-060.anm.37655). Written informed consent to participate

in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Animal Experiments Inspectorate (approval number 2020-15-0201-00549).

AUTHOR CONTRIBUTIONS

DW, LD, LM, JF, EH, EC-L, DC, JT, and CA designed the experiments. DW, SH, LD, and SP performed the experiments. DW, SH, EN, LD, SP, LM, DC, JT, and CA performed data analysis and interpretation. DW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.853482/full#supplementary-material

Supplementary Figure 1 | Whole-mount detection of human Leydig cells into recipient mouse seminiferous tubules following xenotransplantation. One PKH67-positive cell (green) indicating one human cell co-stained with Leydig cell marker CYP17A1 (red) was found within the recipient seminiferous tubule. The nuclei were stained by DAPI (blue). Scale bar, 50 μm.

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Testicular Tissue Banking for Fertility Preservation in Young Boys: Which Patients Should Be Included?

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Due to the growing number of young patients at risk of germ cell loss, there is a need to preserve spermatogonial stem cells for patients who are not able to bank spermatozoa. Worldwide, more and more clinics are implementing testicular tissue (TT) banking programs, making it a novel, yet indispensable, discipline in the field of fertility preservation. Previously, TT cryopreservation was predominantly offered to young cancer patients before starting gonadotoxic chemo- or radiotherapy. Nowadays, most centers also bank TT from patients with non-malignant conditions who need gonadotoxic conditioning therapy prior to hematopoietic stem cell (HSCT) or bone marrow transplantation (BMT). Additionally, some centers include patients who suffer from genetic or developmental disorders associated with prepubertal germ cell loss or patients who already had a previous round of chemo- or radiotherapy. It is important to note that the surgical removal of TT is an invasive procedure. Moreover, TT cryopreservation is still considered experimental as restoration methods are not yet clinically available. For this reason, TT banking should preferably only be offered to patients who are at significant risk of becoming infertile. In our view, TT cryopreservation is recommended for young cancer patients in need of high-risk chemo- and/or radiotherapy, regardless of previous low-risk treatment. Likewise, TT banking is advised for patients with non-malignant disorders such as sickle cell disease, beta-thalassemia, and bone marrow failure, who need high-risk conditioning therapy before HSCT/BMT. TT retrieval during orchidopexy is also proposed for patients with bilateral cryptorchidism. Since patients with a medium- to low-risk treatment generally maintain their fertility, TT banking is not advised for this group. Also for Klinefelter patients, TT banking is not recommended as it does not give better outcomes than a testicular sperm extraction later in life.

Keywords: immature testicular tissue banking, prepubertal boys, fertility preservation, male infertility, spermatogonial stem cells, germ cell loss, testis, (non-)malignant disease

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INTRODUCTION

Over the last decades, the overall rate of childhood and adolescent cancer incidence has increased. The death rates, on the other hand, have strongly decreased due to major advances in oncological treatments. Nowadays, long-term survival is expected in about 80% of young cancer patients (1). In Europe, about 35,000 new cases of childhood and adolescent cancer are reported each year, leading to nearly half a million cancer survivors in 2020 (2). Because of the increase in childhood cancer survivors, an increasing number of adults will have to deal with the long-term effects of cancer treatments. A major complication is the damage to the germ cells and testicular somatic cells caused by the gonadotoxic properties of chemo- and/or radiotherapy. Depending on the survival and functionality of these cells, the testicular damage can result in temporary or even permanent infertility (3). The severity of the testicular damage depends on the type and cumulative dose of chemotherapeutics, the duration, site of irradiation, and fractionation schedule of radiotherapy (4–6). In addition, the patient's age and his individual sensitivity to the gonadotoxic treatment will further determine the fertility outcome (7). Nowadays, gonadotoxic treatments are also used as conditioning therapy for children with non-malignant disorders (e.g., sickle cell disease (SCD) and thalassemia) in need of a bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) (8). To prevent infertility, fertility preservation is often recommended before starting high-risk gonadotoxic treatment in both malignant and non-malignant patients.

The first line method in male fertility preservation is the collection and cryopreservation of mature spermatozoa. However, this method is only applicable when the patient can produce a sperm sample. For pubertal patients with active spermatogenesis who cannot provide a sperm sample by masturbation, assisted ejaculation techniques such as penile vibratory stimulation or electroejaculation under general anesthesia can be used to retrieve sperm. Alternatively, sperm can be obtained from the testicle *via* testicular sperm extraction (TESE) (9). If these techniques are not successful, or not possible because spermatogenesis did not start yet, testicular tissue (TT) can be harvested and banked to preserve the spermatogonial stem cells (SSCs) (10). For prepubertal patients, TT banking is the only available option for fertility preservation.

Since the first experimental fertility preservation program for (pre)pubertal boys in 2002, more centers around the world have started to collect and cryopreserve immature TT from boys at risk of losing their SSCs (10). An international survey conducted in 2019 showed that testicular tissue from more than 1,000 (pre) pubertal boys was banked, which is a 4-fold increase compared to a first survey in 2012 (10, 11). This finding highlights the increasing efforts and acceptability of immature TT cryopreservation programs. The 2019 survey also demonstrated that the indications for TT banking have been expanding since 2012. Previously, most centers almost exclusively offered TT banking to young cancer patients before their cancer treatment (10). Today, the majority also includes patients with non-malignant diseases at risk of germ cell loss, and some include patients who already had a previous round of chemo- and/or radiotherapy. TT banking is also offered to patients with certain genetic or testicular disorders (e.g., Klinefelter syndrome (KS) and cryptorchidism) that are associated with testicular failure leading to fertility problems (11).

The three restoration methods that are currently under development are 1) auto-transplantation of the thawed TT, 2) auto-transplantation of isolated SSCs from the TT, and 3) *in vitro* spermatogenesis (11, 12). Recently, offspring were achieved in non-human primates after successful prepubertal TT grafting (13). When translating this technique to the clinic, the risk of reintroducing tumor cells should be taken into account. Therefore, this method is not recommended for patients who suffered from childhood hematological cancers or metastatic malignancies. For these patients, SSC propagation and auto-transplantation of SSCs would be a better option as the malignant cells could be eliminated during the cell sorting process (7). Although non-human primate embryos (14) and two generations of healthy mouse offspring (15, 16) were obtained after SSC transplantation, the SSC sorting steps and propagation still need further optimization before clinical application is possible. Alternatively, *in vitro* spermatogenesis is proposed to avoid reintroducing malignant cells in a cured patient (11). However, to date, complete *in vitro* spermatogenesis is only achieved in rodents (17) and further culture improvements are needed to achieve safe and efficient human *in vitro* spermatogenesis (18).

Despite the progress that has been made over the past few years, TT banking remains an experimental method as these fertility restoration techniques are currently not yet implemented in clinical practice (19, 20). Moreover, to collect TT, the patients must undergo an invasive biopsy procedure. Although this procedure is shown to have a low short-term complication risk of 2-3% (21–25), only a few studies investigated the possible adverse effects in the longer term. In the prospective study of Uijldert et al. from 2017 (23), it was shown in 64 boys that, up to one year after surgery, the testicular growth of the biopsied testis was similar to the contralateral non-biopsied testis. This finding was confirmed in the prospective study of Borgström et al. (26) in which 21 (pre)pubertal boys, who underwent a TT biopsy before HSCT, were followed for 5.0 to 13.7 years. During this follow-up, the follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were within normal limits for age and pubertal stage in the fourteen surviving patients. However, at the last visit, nine showed high FSH levels, and three showed high LH levels. Besides this, seven out of nine patients showed subnormal anti-Müllerian hormone (AMH) and inhibin B (INHB) levels during the follow-up. This study concluded that these deviating hormone levels were due to the conditioning therapy before HSCT rather than the biopsy procedure itself. Therefore, they concluded that the biopsy procedure had no long-term risks. Reproductive hormone levels were also analyzed in a retrospective cohort study by Kanbar et al. (27). Of the 57 (pre)pubertal boys who underwent TT banking before or after

gonadotoxic treatment initiation, 37% had abnormal gonadotropin levels, 4% had hypogonadotropic hypogonadism, and 33% had primary gonadal failure. These results are in line with previous reports on hypogonadism in childhood cancer survivors (28–31). Therefore, they concluded that a unilateral TT biopsy of <5% of tissue during childhood does not appear to adversely affect the reproductive health outcomes. Despite these reassuring first results, more long-term prospective case-control studies are needed to guarantee the safety of the TT banking procedure.

All taken together, TT banking remains a relatively new technique of which many aspects still require further investigation. To safeguard the well-being of the young patients, and to avoid unnecessary surgery, strict selection criteria are required for this procedure. In this review, the risk of infertility for the most frequent conditions and treatments will be described based on the most recent findings. Moreover, as the indications for TT banking vary between centers, this review aims to revise the current inclusion criteria and gives advice on which patients are (still) eligible for TT banking.

MALIGNANT DISEASES

The most common indication for TT banking continues to be childhood malignant disease. Within this category, the most prominent disorders are hematological cancers, central nervous system tumors, Ewing family tumors, rhabdomyosarcoma, osteosarcoma, neuroblastoma, and other non-central nervous system solid tumors (11). Malignancies as testicular cancer, extragonadal germ cell tumors, Hodgkin's disease, and leukemia are associated with a reduced gonadal function, even before the start of the treatment (32–40). The reproductive health of cancer patients can be affected through various mechanisms. Firstly, the cancer itself can disrupt the hypothalamic-pituitary-gonadal (HPG) axis in the patient, which is fundamental for the reproductive hormone production and later fertility. Some tumors produce endocrine substances like beta-human chorionic gonadotropin or alpha-fetoprotein, that trigger negative feedback on the HPG axis (41–44). Other cancers like leukemia, lymphoma, or central nervous system tumors directly impair the pituitary gland through tumor cell invasion (45) Secondly, some tumors directly impair the testicular function by invading the healthy testicular tissue and causing local damage to the cells (5). Thirdly, the pro-inflammatory response and other effects of the malignancy (e.g., pain, fever, and anorexia) may also impair the patient's fertility (34, 36, 45, 46). Lastly, the cancer diagnosis itself may affect the psychological wellbeing of the patient, potentially causing an additional negative impact on spermatogenesis and sexual function in adolescence and adulthood (47–49).

Although the malignancy itself plays a role, the fertility decline in cancer patients predominantly depends on their treatment with surgery, chemotherapy, local or total body irradiation, and/or a combination of those. The cancer treatment can either cause direct damage to the germ cells and/or the somatic cell populations of the testis (3), or it can have indirect effects through damage to the HPG axis (50). Contrary to previous findings, recent evidence indicates that gonadotoxic therapy can impair the gonadal function at any pubertal stage (51, 52). It has even been shown that the prepubertal testis is more susceptible to the damaging effects of gonadotoxic agents than adults because of the constant turnover of Sertoli cells and early germ cells (53, 54). Production of mature spermatozoa during puberty is determined by the number of surviving SSCs and the functionality of the somatic cell populations. A drastically reduced SSC pool may require several years before spermatogenesis recovers. However, a completely depleted SSC pool will result in permanent infertility (3, 55, 56). The risk for infertility depends on the type and dose of administered agents and the combination of the received cytotoxic therapy (7). The major cytotoxic treatment regimens are therefore categorized into low-, medium- and high-risk groups (5, 57, 58). The degree of gonadal damage for the different treatment modalities will be discussed in the following sections.

Surgery

Tumor resection surgery involving the reproductive organs can affect the patient's fertility potential. Performing a unilateral orchiectomy in patients with testicular cancer results in decreased germ cell numbers, and may put them at risk for prematurely reduced Leydig cell function (59–61). In these patients, both the cancer itself and the surgical intervention can cause impaired spermatogenesis (62). Most types of central nervous system tumors require tumor resection, which may cause damage to the hypothalamus and/or pituitary glands, leading to gonadotropin deficiencies (63). In some cases, chemo- and/or radiotherapy can be used to circumvent a surgical intervention in the gonads or hypothalamus-pituitary region. However, these treatments could in turn also increase the risk for gonadal damage (1).

Chemotherapy

Chemotherapy is one of the most commonly used treatments to cure cancer. Its principle is to target the rapidly dividing cancer cells. Nevertheless, chemotherapy does not only target the cancerous cells, it also targets other healthy dividing cell populations (64). Sertoli cells are also actively dividing during childhood, making them an additional target for cytotoxic agents (3). Although limited data are available on the long-term impact, Sertoli cell dysfunction was reported following chemotherapy exposure during infancy (65, 66). Leydig cells seem more resistant to chemotherapeutic agents as reports on androgen insufficiency following chemotherapy alone are uncommon (3, 67).

Chemotherapeutic agents can be categorized as low-, medium-, or high-risk depending on their expected degree of gonadal damage. **Table 1** gives an overview of frequently used chemotherapeutic agents and their impact based on previous publications (5–7, 58, 68, 69). The high-risk group consists of high-dose alkylating and platinum-based agents that are associated with a >80% risk of infertility (5, 56, 71). The medium-risk group mainly contains low-dose alkylating and

TABLE 1 | Estimated risk of prolonged azoospermia with chemo- and radiotherapy.

	High risk (indication for TT banking)	Medium risk	Low risk
Chemotherapy	Busulfan >600 mg/m² (5, 7, 58) Carmustine 1 g/m² (5–7, 69) Chlorambucil >1.4 g/m² (5–7, 58, 68, 69) Chlormethine UD (7, 58, 69) Cisplatin >600 mg/m² (5–7, 68, 69) Cyclophosphamide >19 g/m² (5–7, 58, 68, 69) Ifosfamide >52 g/m² (5–7, 58, 68) Lomustine 500 mg/m² (if treated before puberty) (5, 6) Mechlorethamine UD (7, 68) Melphalan >140 mg/m² (5–7, 58, 68, 69) Mustine UD (7) Procarbazine >4 g/m² (5–7, 58, 68, 69)	Carboplatin >2 g/m² (5, 7, 58, 68) Cisplatin 400–600 mg/m² (5, 7, 58) Cyclophosphamide 7.5-19 g/m² (5) Cytarabine 1 g/m² (7, 68) Dacarbazine UD (7, 69) Daunorubicin UD (7, 69) Doxorubicin >770 mg/m² (5–7, 58, 68, 69) Gemcitabine UD (7, 69) Ifosfamide 42–52 g/m² (5) Mitoxantrone UD (7, 69) Oxaliplatin UD (7, 68, 69) Thiotepa 400 mg/m² (5, 7, 69)	Actinomycin-D UD (5, 7) Azathioprine UD (7, 69) Bleomycin UD (5–7, 58, 68, 69) Cytarabine <1 g/m² (5) Dactinomycin UD (58, 68) Etoposide UD (5–7, 68, 69) Fludarabine UD (7, 69) 5-Fluoracil UD (5, 6, 69) 6-Mercaptopurine UD (5–7, 58, 68, 69) Methotrexate UD (5, 6, 58, 68, 69) Vinblastine 50 g/m² (5–7, 58, 68, 69) Vincristine 8 g/m² (5–7, 58, 68, 69)
Radiotherapy	Total body irradiation (5, 7, 58) Testicular radiotherapy (5, 7, 58, 70)	Craniospinal- and cranial radiotherapy ≥25 Gy (5, 58, 70) Scattered abdominal or pelvic radiation ≥1 Gy (5)	Lower radiation doses

UD, undetermined dose.

platinum-based agents, anthracyclines, and antimetabolites. The compounds in this group give an estimated risk of 20-80% on infertility (5). The low-risk group consists of vinca alkaloids, certain antimetabolites, and non-anthracycline antibiotics (6, 68, 72). Low-risk agents generally induce a short-term interruption of spermatogenesis with a <20% risk of prolonged azoospermia (5).

It is important to note that high cumulative doses and the combination of different cytotoxic agents can significantly increase the risk for long-term fertility problems (73). Besides this, it remains a challenge to identify the individual risk per molecule, as these are usually administered in combinations (58, 67, 69, 74). Moreover, most data either come from animal experiments or studies performed in adult men, which makes it difficult to extrapolate these risks to young patients (67, 75, 76). As the exact risk classifications/dosages for children remain unclear, further research on prepubertal exposure is warranted together with regular updates of this classification (67, 68).

Radiotherapy

Another frequently used cancer treatment is radiotherapy. Analogous to chemotherapeutic agents, radiation targets the cancerous cells but has also off-targets like the gonads. The testes are one of the most radiosensitive tissues of the male body (72). Damage induced by radiotherapy is highly dependent on the total dose, duration, the irradiation field, and the fractionation schedule (77). Fractionated radiation usually diminishes the side effects, however, the testicular tissue seems more sensitive to fractionated therapy as it reduces the time available for repair (58, 72). Testicular radiation doses of 0.1-1.2 Gy can result in temporarily oligo- or azoospermia. Doses of 2-3 Gy affect the SSCs, causing long-term infertility in the patient. Doses of 6 Gy and more completely deplete the SSC pool and lead to permanent azoospermia (78–80). Total body irradiation (TBI), used as conditioning therapy before BMT or HSCT, is also linked to gonadal dysfunction (81).

Testicular irradiation and TBI with doses of 10-12 Gy have been reported to harm Sertoli cells. The damage to the Sertoli cell is linked with smaller testicular volumes at adulthood and high FSH and low INHB levels (53, 82, 83). Recent research indicates that Sertoli cells are the most radiosensitive just before the start of spermatogenesis. This is probably due to the high proliferation rate of the Sertoli cells in the testis during early puberty (3, 82, 84). Leydig cells are generally more resistant to irradiation damage. Leydig cell dysfunction and testosterone deficiency are usually undetected up to doses of 30 Gy in adult men and 20 Gy in prepubertal boys (4, 73, 85). This might be attributed to a compensatory increase in LH levels that corrects testosterone production, leading to a normal puberty initiation despite the gonadal damage (3, 58).

Impaired fertility has also been reported after cranial radiotherapy using doses of 35–45 Gy. Cranial radiotherapy does not directly damage the gonads, but it is known to cause neuro-endocrine imbalances when the HPG axis lies within the

irradiation field (86). This might cause gonadotropin deficiencies, leading to decreased sex steroid levels and resulting in impaired spermatogenesis and delayed puberty (87). When the gonadotropin deficiency is only caused by damage to the HPG axis, it can be treated by administration of exogenous gonadotropin-releasing hormone (GnRH) or gonadotropins (58). However, when there is additional gonadal damage, the problem could persist (88). Cranial radiotherapy with doses of 25 Gy and more can, paradoxically, also cause early puberty by prematurely activating the HPG axis. This can be resolved by the administration of GnRH analogs (89).

As shown in **Table 1**, a classification can be made based on the risk for later fertility problems. The high-risk class (>80%) consists of TBI and testicular radiotherapy (5, 58). Analogous to chemotherapy, high-risk treatment is restricted as much as possible in current pediatric treatment protocols because of the late adverse effects (1, 90, 91). Some papers only consider testicular irradiation with doses of 6 Gy and more as high-risk (5, 92). Nevertheless, because even very small doses of testicular irradiation can cause irreversible damage to the SSCs (78, 79), any dose should be considered as a risk (58, 70, 73). Craniospinal- and cranial irradiation doses of 25 Gy and more as well as testicular irradiation of 1-6 Gy using scattered abdominal or pelvic radiation should be considered as medium-risk therapy (20-80%) (5, 58). Lower radiotherapy doses are classified as low risk (<20%).

Targeted Therapy

Targeted cancer treatments are gaining popularity in the field of oncological research and clinical application. In contrast to conventional chemo- and radiotherapy, targeted therapies will specifically act on the cancerous cells instead of all rapidly dividing cells (93). Many targeted cancer therapies are currently being studied in (pre)clinical trials and several have already been approved for specific cancer types by the Food and Drug Administration (FDA) (94). Tyrosine kinase inhibitors, for example, are nowadays indispensable in the treatment of childhood chronic myeloid leukemia and acute lymphoblastic leukemia. Nevertheless, only limited data are available on the impact of targeted therapy on male fertility and more research is needed on this topic (32). The effects of FDA-approved targeted cancer therapies on male fertility that are already known are summarized in a recent review (19). Briefly, the use of Imatinib, Dasatinib, Everolimus, Gemtuzumab, and Voxelotor was associated with some adverse effects on the reproductive health in rat studies. Moreover, Imatinib was found to decrease the sperm count in clinical adult human studies and a case report showed severe oligozoospermia when Imatinib was taken during prepuberty. Interestingly, short-term Imatinib treatment in prepubertal rats did not cause infertility nor affect the offsprings' health. For Nilotinib, Entrectinib, Larotrectinib, Selumetinib, Avelumab, Nivolumab, Rituximab, Dinutuximab, Blinatumomab, Pembrolizumab, Ipilimumab, Tisagenlecleucel, and Tagraxofusp, no adverse effects on male fertility were found so far. However, more studies on the impact on the prepubertal testis and later spermatogenesis are required to verify these first results.

NON-MALIGNANT DISEASES

Nowadays, HSCT is not only a curative treatment option for patients with malignant hematological diseases, but also for patients with SCD, thalassemia, bone marrow failure, or other non-malignant conditions (11). HSCT requires conditioning therapy with high-risk chemotherapy and/or TBI. Therefore the patients are at risk for long-term side effects such as impaired pubertal development and/or impaired fertility (3, 8, 74, 95–97). In some cases, previous treatment or the disease itself could have caused spermatogonia loss (74). Young boys treated for non-malignant diseases are also suitable candidates for TT banking. The infertility risks for the most prominent diseases will be further elaborated in the following paragraphs.

Sickle Cell Disease

SCD or sickle cell anemia is the most common non-malignant condition for which allogeneic HSCT is recommended (74). Each year, more than 250,000 children are born with this condition (98). According to the questionnaire of Goossens and colleagues (11), SCD is the main indication for immature TT banking within the benign diseases. SCD is an autosomal recessive disorder in which abnormal hemoglobin S is created that will form polymers when deoxygenated. This gives the red blood cells their characteristic sickle shape, leading to chronic hemolysis and vaso-occlusion (99, 100). Due to tissue hypoxia, multiple organs can be damaged throughout the patient's life and common complications are severe chronic pain, chronic organ failure, stroke, acute chest syndrome, infections, and early mortality (101–103).

For many years, it is known that the SCD itself can cause delayed puberty as well as disturbed semen parameters and sperm abnormalities at adulthood (104–106). The most common cause for infertility in SCD men is hypogonadism. Furthermore, fertility problems can also be linked to erection disorders, sexual problems, testicular ischemia/infarction, zinc deficiency, and small testicular size (107–109). In addition to the disease itself, many of its primary treatments are associated with fertility impairment. SCD patients are often prescribed opioids and analgesics to ease their chronic pain (99). In general, opioids can decrease the levels of reproductive hormones like LH, testosterone, and estradiol. Chronic opioid use is shown to cause hypogonadism and could also disturb the secretion of other pituitary hormones (110). SCD patients require frequent blood transfusions to control their anemia (99). These transfusions can cause severe iron accumulation, which can be disposed in several organs, including the pituitary gland and testicles. This could cause hypogonadism and damage the testicular cells, leading to impaired fertility (32). To prevent or treat this iron toxicity, patients often take iron chelators to remove the iron excess. However, intensive iron chelation therapy is suspected to suppress spermatogenesis (111).

Hydroxyurea (HU) therapy is another commonly used treatment to prevent vaso-occlusive pain episodes, acute chest syndrome, hospital admissions, and the need for blood transfusions in SCD patients. HU is an FDA-approved non-alkylating antineoplastic agent that can increase the

concentration of fetal hemoglobin and thus prevent the sickling of the red blood cells with the mutated adult hemoglobin (107, 112). One of the main consequences of HU is that it seems to worsen the semen parameters in adults (107). To date, only a few studies are available regarding the effect of HU treatment on the number of spermatogonia in prepubertal patients, and the results are contradictory. Some studies have reported that the spermatogonial pool is drastically reduced or even fully depleted following HU treatment (25, 74, 113). More recent studies, however, suggest that HU therapy might not be the main cause of the spermatogonial loss and fertility problems in SCD patients with severe genotypes (114–116). Nevertheless, as the studies on HU did not always include an untreated control group, the effects of SCD itself are difficult to distinguish from those of the HU therapy. Most of the studies only contained a limited number of patients, making it difficult to draw firm conclusions. Larger follow-up studies are needed to verify whether: 1) the spermatogonial loss and disturbed semen parameters are directly caused by the HU treatment or SCD itself or a combination of both, 2) there is a correlation between the timing and duration of HU treatment and the severity of fertility impairment, and, 3) the effects are (partially) reversible after cessation of the HU therapy (107, 108, 115, 117). HU is an antimetabolite classified within the medium-risk group (**Table 1**). For this reason, TT banking is not advised as long as the patient is not eligible for HSCT.

For patients with a severe SCD phenotype and end-organ damage, allogeneic HSCT is recommended (117, 118). This treatment option does involve side effects like risk for rejection, transplant-related mortality, and chronic graft-versus-host disease (115). Certain immunosuppressants that are administered for transplantation are associated with hypogonadism and reduced male fertility (119). Nonetheless, the biggest impact on their fertility is caused by the myeloablative conditioning therapy they have to undergo before HSCT. This conditioning regimen typically consists of high doses of alkylating agents such as cyclophosphamide and busulfan and/or TBI. As summarized in **Table 1**, these treatments all belong to the high-risk category and can cause endocrine dysfunction and impaired fertility (108, 120). Moreover, patients with SCD have been shown to already have a reduced number of spermatogonia before HSCT (74, 103, 114). Because of this, TT banking is recommended for these boys before they start high-risk therapy.

Beta-Thalassemia

Beta-thalassemia is the second most common hereditary hematological disorder, with about 60,000 new symptomatic cases each year (98). Beta-thalassemia is an autosomal recessive disease in which the production of beta-globin chains, that constitute adult hemoglobin, is disturbed (121). This defect leads to ineffective erythropoiesis and hemolysis in the bone marrow or spleen (122). In beta-thalassemia intermedia (B-TI), the beta-globin chain synthesis is severely reduced. The most severe form of the disease is transfusion-dependent thalassemia, better known as beta-thalassemia major (B-TM). In B-TM patients there is no beta-globin chain synthesis, leading to serious hemolysis, anemia, and hypoxia in several tissues, including the testis (122, 123).

B-TM patients need lifelong blood transfusions to avoid skeleton deformities, hepatomegaly, and splenomegaly (121). As discussed in the SCD section, blood transfusions can initiate iron build-up and severely affect several organs, including the pituitary gland and testicles, causing hypogonadism and testicular tissue damage (32, 123, 124). Moreover, the iron overload is usually worse in B-TM patients as their excessive hemolysis exacerbates the iron release. This is also a reason why B-TM patients are generally more affected than B-TI patients (63, 103). Leptin synthesis can also be decreased in B-TM patients due to iron accumulation in the adipose tissue, further impairing sexual maturation and fertility (125). As for SCD patients, iron chelation therapy can minimize the iron accumulation but may impair spermatogenesis (111). Besides this, decreased seminal and plasma zinc levels are not uncommon in these patients (126). Due to a combination of all the above-mentioned effects, delayed puberty and fertility issues are common findings in B-TM males (103, 124, 126–129).

Despite the declined morbidity and mortality with periodic blood transfusion and iron chelation, HSCT remains the only curative option for patients with B-TM (103). Transplanted patients generally have a better long-term life quality than patients receiving chronic blood transfusions and chelation therapy (123). Nevertheless, transplanted patients are known to develop secondary complications from their high-risk myeloablative conditioning therapy and HSCT, including hypogonadism and infertility, as previously discussed (123, 129). As with SCD, patients with B-TM may have a reduced number of spermatogonia, already before undergoing HSCT (74). Therefore, it is advocated to preserve TT in B-TM patients before they start high-risk conditioning treatment, which depletes the SSC pool even further (124). Previous research showed that more adverse effects are visible in patients who underwent transplantation at an older age (>15 years) or patients who did not receive sufficient chelation therapy before transplantation (130, 131). Nonetheless, since the conditioning therapy still poses a very high risk of infertility, and it is currently impossible to predict with certainty which patients will remain fertile, it is counseled to bank immature TT tissue regardless of age at transplantation.

Bone Marrow Failure

Childhood bone marrow failure (BMF) is another well-represented group of benign conditions for which immature TT is banked (11). The term BMF encompasses a heterogeneous spectrum of diseases in which the patient has impaired hematopoiesis in the bone marrow, causing inadequate production of one or more circulating blood cell lines (erythroids, myeloids, and/or platelets) (132). BMF can both be acquired (e.g., aplastic anemia and paroxysmal nocturnal hemoglobinuria) or congenital (e.g., Fanconi anemia and dyskeratosis congenita). Aplastic anemia is the most common syndrome on this list (132, 133). Clinical manifestations of these disorders are anemia, neutropenia, and/or thrombocytopenia, and may be associated with hemolysis and thrombophilia (134).

Similar to the previously discussed blood disorders, first-line treatment often consists of blood transfusions to counteract the anemia, as well as chelation therapy to limit iron overload. Platelet transfusion can be administered to prevent or treat bleeding due to thrombocytopenia (132). In the case of acquired aplastic anemia, a condition associated with autoimmunity, immunosuppressive therapy with antithymocyte globulin and cyclosporine A is used (132, 135, 136). So far, this therapy has not been associated with reduced fertility in women, but the exact effect on male fertility has yet to be explored (137). However, as with most benign blood disorders, HSCT can be offered to patients with a severe form of BMF to restore the function of the hematopoietic stem cells. This is often the case in patients with Fanconi anemia or (very) severe aplastic anemia to improve their survival rates (138). The standard conditioning regimen for these diseases contains high-dose cyclophosphamide, inducing a high risk for gonadal dysfunction as shown in **Table 1** (132, 138). Hence, it is advised to store immature TT of these patients before the start of conditioning therapy.

Fanconi anemia often causes genital abnormalities as cryptorchidism, hypospadias, seminiferous tubule hypoplasia, and small testes. The disease itself is also shown to affect the HPG-axis, with hypogonadism, delayed or accelerated puberty, and fertility issues as previously discussed (139, 140). These patients already demonstrate spermatogonia loss before gonadotoxic treatment and adults have extremely low sperm concentrations (74, 139). For these reasons, fertility preservation in young Fanconi anemia patients undergoing HSCT is even more crucial.

Others

Some other benign pathologies for which TT banking is recommended because of the fertility problems linked to high-risk treatment are inborn errors of metabolism for which other available therapies are less effective, and severe immune diseases that are not responsive to immunotherapy (11, 141). Well-known inborn errors of metabolism for which HSCT can be useful are lysosomal storage diseases, peroxisomal disorders, and mitochondrial diseases (141). Kostmann syndrome, chronic granulomatous disease, and Wiskott-Aldrich syndrome are some examples of immune diseases for which HSCT therapy may be applied (32, 142, 143). Additionally, high-risk agents such as cyclophosphamide are often used to suppress the immune system in patients with juvenile-onset systemic lupus erythematosus (144).

GENITAL, TESTICULAR, AND SEXUAL DISORDERS

The third and last category of childhood conditions that may affect fertility and for which immature TT has been banked over the last years consists of genital, testicular, and sexual disorders (11). As this category contains markedly fewer patients than the previous ones, only the two most frequent indications will be explained in more detail.

Cryptorchidism

Undescended testis or cryptorchidism is a pediatric condition in which one (unilateral cryptorchidism) or both testes (bilateral cryptorchidism) fail to descend into the scrotum. Cryptorchidism is one of the most common congenital anomalies that affects about 1-4% of full-term and 30% of preterm newborns worldwide (145). This condition is associated with hormonal defects, testicular torsion, inguinal hernia, and an increased risk of testicular germ cell tumors (146, 147). When the testes are absent in the scrotum during the first years of life, the number of germ cells will also drastically decrease, impairing future fertility (148). Previous research showed that around 13% of patients with unilateral cryptorchidism and up to 89% of patients with untreated bilateral cryptorchidism will be azoospermic later in life (149). Although the exact cause of the infertility and testicular malignancy is still unknown, researchers assume that the higher environmental temperature causes heat stress, resulting in abnormal transformation and excessive cell death of neonatal germ cells (149–151).

Some researchers suggested that hormone therapy with human chorionic gonadotrophin, GnRH, or LH-releasing hormone could have an additional protective effect on the fertility of boys with cryptorchidism. However, the results of these studies are not convincing and therefore, hormonal therapy is not advised (152). To decrease the risk of infertility and malignancy, orchidopexy is recommended to permanently anchor the testis into the scrotum. According to the American and European urology associations, this surgical procedure should be performed between 6-18 months of age as spontaneous testis descendance hardly occurs after 6 months of age (146, 153, 154). Besides this, later orchidopexy has been shown to reduce the testicular volume and the number of germ cells and to increase the risk of malignancy and infertility (154–157).

Nevertheless, the risk of azoospermia remains 18–46% in patients with bilateral cryptorchidism despite early surgical treatment (149, 158). To preserve the fertility of these patients while germ cells are still present in their testes, TT could be harvested for long-term storage during the orchidopexy procedure or during a separate procedure (92, 159, 160). This cryopreservation procedure is generally well accepted by the parents (161). Since patients with unilateral cryptorchidism generally have a low risk of becoming infertile, TT cryopreservation is not recommended. For patients with bilateral cryptorchidism, it is proposed to harvest TT during the orchidopexy procedure because they have a higher risk of later infertility. However, retrieving TT during a separate biopsy procedure is not recommended as this would require another invasive surgery procedure (160).

Klinefelter Syndrome

With an incidence of as much as 1-2/1,000 newborn males, Klinefelter syndrome (KS) is the most frequent sex chromosome abnormality in the human (162). About 80% of the KS patients have a 47, XXY karyotype and the other 20% carry higher grade aneuploidies or mosaicisms (163). The phenotype of KS is highly variable and is generally worse in patients with a 47,XXY

karyotype or higher-grade aneuploidies compared to mosaic patients (164). Typical KS symptoms are a tall and feminine posture, gynecomastia, neurocognitive and psychosocial problems, cryptorchidism, small firm testes, and hypogonadism (165, 166). Moreover, KS is characterized by germ cell loss already in early childhood and from puberty onwards testicular fibrosis, degeneration of the seminiferous tubules, and Leydig cell hyperplasia (167, 168). More than 90% of the non-mosaic KS patients are diagnosed with azoospermia later in life (169).

With this in mind, TESE during early adolescence has been proposed in the hope of having better success rates compared to TESE in adulthood (170–172). The rationale behind this was to perform the TESE procedure before the testicular tissue degeneration and before testosterone supplementation which could further suppress spermatogenesis (173). However, to this date, no proof shows that a biopsy during prepuberty or a TESE during adolescence is more successful than a TESE during adulthood (167, 173–175).

In order to perform fertility preservation before germ cell loss, KS patients have also been enrolled in several experimental immature TT banking programs (96, 167, 176, 177). However, a lot of the biopsied testicular tissues were found to be sclerotic, making fertility restoration methods, including auto-transplantation and *in vitro* spermatogenesis (IVS), unfeasible. Additionally, as the biopsies only rarely contained spermatogonia and the TT biopsy procedure may further reduce the number of spermatogonia, TT banking in KS patients remains a highly controversial subject (11, 167, 178). It is important to note that the children and adolescents included in these studies (96, 167, 176, 177) are a different population than the adults who were included in earlier TESE studies. It could therefore be possible that these children and adolescents also score poorly with a TESE when they are grown up. More longitudinal research is required to validate this.

As TESE outcomes at adulthood are comparable to those at adolescent age (179), it is reasonable to wait until the patient has a concrete childwish. Therefore, immature TT banking is not recommended for KS patients outside research programs, and TESE during early adulthood should remain the first line fertility preservation method (169, 180, 181).

Others

Other developmental and acquired conditions such as testicular torsion, varicocele, reproductive tract infections, hypospadias, ambiguous genitalia, benign tumors, spina bifida, and congenital adrenal hyperplasia are also associated with male infertility (182). As most of these conditions can be surgically treated during childhood or adulthood, these patients generally do not face a high risk of infertility and immature TT is not frequently banked (11).

DISCUSSION

Since 2002, fertility centers are offering immature TT banking to young patients who are at high risk of losing their SSCs (22, 25, 159, 183–185). Initially, centers only cryopreserved TT from cancer patients at significant risk of infertility due to the gonadotoxic properties of chemo- and radiotherapy (10). Gradually, an increasing number of centers is offering fertility preservation, resulting in expanding inclusion criteria (11). However, as TT banking is still experimental and restoration methods are not yet clinically performed, it is of great importance that TT banking is currently only offered to patients at high risk of infertility and for whom the advantages of this experimental procedure outweigh its potential risks (11, 20). Therefore, this review provides advice on which pediatric patients could benefit from TT banking.

Concerning children with malignant diseases, previous research has shown that most parents agree to preserve TT, even with a low risk of later fertility problems and an unknown success rate of future fertility restoration techniques (21, 186–189). Despite these findings, we agree with the recent guidelines of the PanCareLIFE Consortium and the International Late Effects of Childhood Cancer Guideline Harmonization Group (70) and only recommend TT banking for young cancer patients undergoing high-risk gonadotoxic treatment. This high-risk group consists of patients who need to undergo high-dose alkylating or platinum-based agents, TBI, testicular radiotherapy, or a combination. In some cases, cancer patients do not receive high-risk therapy unless they show a weak response to first-line low-risk chemotherapy or after relapse (73, 97). Since previous findings have shown that the SSC pool remains within the normal range after the first-line low-risk treatment, these patients should also be offered TT cryopreservation before the initiation of the high-risk treatment (71, 74, 190). However, additional research should be performed to verify whether the quality of the SSCs is still sufficient for fertility restoration after chemotherapy. When considering methods of fertility restoration, the risk of contamination of the biopsy with malignant cells must also be taken into account. To avoid this, tissue auto-transplantation is not recommended, and SSC transplantation after depletion of the malignant cells should be the preferred method. Nonetheless, before the latter would be clinically applicable, SSC purification techniques need to be fine-tuned (11, 12). Alternatively, IVS followed by ICSI could be proposed, but this technique still requires extensive research before it can be applied in the clinic (11).

Because of the invasive and experimental nature of the procedure to date, immature TT banking is not recommended for cancer patients with a medium- to low-risk treatment (70). Importantly, Kanbar et al. recently confirmed that these patients generally maintain their fertility, making TT banking redundant (27). However, as chemotherapeutic agents are mostly administered in combination, it is difficult to assess the individual risk per agent (58, 67, 69). Moreover, most results are extrapolated from animal or adult studies, making additional research necessary to verify these risk classifications/dosages in children (67, 68, 75, 76, 191). Therefore, studies with human-relevant experimental systems such as human organoids (18) or human organotypic culture combined with xenografting (192), could be helpful tools to characterize the exact effects of a

chemotherapeutic agent on prepubertal testicular tissue. Besides this, FDA-approved targeted cancer treatments are not expected to cause a high risk of infertility, but further studies on long-term reproductive effects are needed to confirm this (19).

Although our risk classification is based on the most recent data available in the literature, it should be handled with caution for the reasons mentioned above. Furthermore, the risk of developing infertility is dependent on several patient-related factors like the patients' age, type of cancer, and comorbidities (193). Consequentially, TT preservation should be advised on an individual basis, especially for patients with complex treatment protocols (194). Several studies demonstrate that the patients and their parents want to be educated about experimental fertility options despite the stressful time at diagnosis (21, 195). Therefore, regardless of the risk of the therapy, clinicians should counsel the patients and their families and inform them as soon as possible about the estimated effects of the therapy on the fertility of the patient (6, 70). The clinicians should be open to discuss possible fertility preservation options and refer the patient and his parents to a reproductive specialist when needed (193). Multi-collaborative care pathways with well-informed oncologists and reproductive specialists are necessary to facilitate the decision-making and increase the patient referrals and acceptance rates of the banking procedure (11, 196).

Analogous to cancer patients, children with non-malignant conditions often need high-risk chemo- and/or radiotherapy as conditioning treatment before HSCT. For this reason, immature TT banking is recommended in this patient group. Moreover, in SCD, thalassemia, and certain BMF patients, the disease itself could already cause some fertility issues. Patients with a FOXP3 gene deficiency or Fanconi anemia were also found to already have a reduced SSC pool prior to gonadotoxic therapy, which makes them even more vulnerable to its toxic effects (74, 139). Besides this, patients with SCD very often receive long-term HU treatment, posing an additional risk for later infertility (197). As none of these patients risk malignant cell contamination, tissue transplantation would be the most promising method for fertility restoration (11, 12).

Patients who suffer from bilateral cryptorchidism still have a relatively high risk for fertility problems, even after early orchidopexy (149, 158). Consequently, TT could be harvested during their recommended orchidopexy procedure while their testes still contain a sufficient number of germ cells (160, 161). Nevertheless, performing a separate TT biopsy procedure for this is not recommended. For Klinefelter patients and patients with other testicular disorders, TT banking is not recommended. This is because most testicular disorders can be treated surgically during childhood, which reduces the risk of infertility. For these patients, the benefits do not outweigh the possible negative effects of a testicular biopsy (11). Although patients with KS do have a high risk of azoospermia during adulthood, taking a TT biopsy is currently not advised as the tissue is often sclerotic and only rarely contains spermatogonia (11, 167, 178). It has been recommended to wait until the patient wishes to have children, as TESE combined with intracytoplasmic sperm injection shows promising results (169, 179–181).

In conclusion, today, immature TT banking is classified as an invasive and experimental procedure whose long-term effects and clinical applications have yet to be thoroughly investigated. Therefore, immature TT banking is only recommended for young cancer patients who need to undergo high-risk chemo- and/or radiotherapy, regardless of earlier low-risk treatment. Likewise, TT banking is advised for patients who need high-risk conditioning therapy before HSCT/BMT to cure their non-malignant disorder (e.g., sickle cell disease, beta-thalassemia, and bone marrow failure). Patients undergoing medium- to low-risk therapy and their parents should be informed about the possible impact of the therapy on the reproductive function, but TT banking is not advised in these patients. It is proposed to retrieve a TT biopsy during orchidopexy in patients with bilateral cryptorchidism. For Klinefelter patients, however, TT banking is not recommended because it generally does not give better outcomes than a TESE later in adult life. Also for patients with other testicular disorders, a TT biopsy is not recommended as the benefits usually do not outweigh the risks.

AUTHOR CONTRIBUTIONS

ED performed the literature search and manuscript writing, while AB and EG contributed to the critical revision of this review. All authors were involved in the conception and design of this review and approved the submitted version.

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Short-Term Hypothermic Holding of Mouse Immature Testicular Tissue Does Not Alter the Expression of DNA Methyltransferases and Global DNA Methylation Level, Post-Organotypic Culture

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Introduction: Cryopreservation of immature-testicular-tissue (ITT) prior to gonadotoxic treatment, while experimental, is the only recommended option for fertility preservation in prepubertal boys. The handling and manipulation of ITT prior to banking could influence the functionality, genetic and epigenetic integrity of cells.

Objectives: To investigate the impact of length of hypothermic holding of mouse ITT on the relative mRNA expression of the DNA methyltransferases (DNMTs) and global DNA methylation, post 14-days of organotypic culture.

Methods: ITT from 6-day old mice were handled at hypothermic temperature (4 °C) for 6 and 24 h prior to 14-days organotypic culture. Relative mRNA expression of *Dnmt1, Dnmt3a*, and *Dnmt3b* along with global DNA methylation was measured from the cultured ITT.

Results: No significant variation in the expression of *Dnmt1, Dnmt3a*, and *Dnmt3b* was observed in relation to varying holding time periods used. Further, global DNA methylation was comparable between 0, 6 and 24 h holding groups.

Conclusions: Short-term holding of ITT at 4 °C does not affect the DNA methylation process post organotypic culture. While fully acknowledging the limitations of this approach in the mouse model, the results we presented in this report will be of significant interest to the field.

Keywords: immature testicular tissue, organotypic culture, hypothermic holding temperature, DNA methylation, epigenetics, DNMT, fertility

INTRODUCTION

Childhood cancer survivors are at risk of experiencing infertility as one of the long-term health complications of cancer therapy. In males, this is due to the potential impact of gonadoxic agents on actively dividing spermatogonial stem cells (SSCs), which provide the foundation for normal spermatogenesis (1–4). To overcome this health hazard, currently, cryopreservation of immature testicular tissue (ITT) is the only available fertility preservation option for prepubertal boys as spermatogenesis is not fully functional in them. However, this approach is still considered experimental (5, 6).

Due to the limited number of centers that offer ITT banking worldwide (5), transporting the tissue from the testicular biopsy site to the tissue banking facility is inevitable. In this regard, studies have determined the optimal conditions for ITT such as tissue size, storage temperatures, and storage periods in various models including human tissue (7–11). Recently, our group has demonstrated that ITT manipulation at 4°C had a minimal negative impact on the organotypically cultured germ cell population when compared to room temperature and 37°C (12). However, we believe that it is important to address the epigenetic integrity of germ cells from cultured ITT as epigenetic aberrations may negatively affect the subsequent developmental process.

DNA methylation is critical for fertilization, embryonic development, and postnatal life (13–17). The family of DNA methyltransferases (DNMT's) consists of *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, responsible for maintenance and *de novo* establishment of methylation patterns on 5'-positions of cytosine on DNA (18, 19). Epigenetic modifications are heritable changes in gene function independent of alterations in DNA sequence (20, 21). Although studies have shown that global DNA methylation level and expression of enzymes responsible for DNA methylation are unaffected during *in vitro* culture (22, 23), the impact of hypothermic holding of ITT prior to the organotypic culture on DNMT's expression and global DNA methylation is not elucidated so far. Hence, using the mouse model, this study was aimed to investigate the impact of hypothermic holding of ITT on the relative mRNA expression of the DNMT's and global DNA methylation post-14-days of organotypic culture.

MATERIALS AND METHODS

Animals, Ethical Clearance, and Testicular Tissue Collection

A total of twenty-four, 6 day-postpartum (dpp) male Swiss albino mice were used in the study. All experiments and animal handling were conducted in accordance with the institutional guidelines for animal experimentation after obtaining prior approval from the Institutional Animal Ethics Committee (Kasturba Medical College & Kasturba Hospital Institutional Ethics Committee, approval #IAEC/KMC/93/2013). Animals were sacrificed by cervical dislocation and the testes were collected in alpha minimum essential medium (α-MEM + Glutamax; (32571-036; Gibco™, Grand Island, USA) containing 1% (v/v) penicillin-streptomycin (Pen-Strep; 15140-122; Gibco™, Grand Island, USA) and 5 µg/mL Nystatin (Nys; N3503; Sigma-Aldrich, St. Louis, USA). Testes were made fat-free using fine needles, under the stereomicroscope, and later randomly distributed/categorized for either holding-phase or direct culture.

Holding Phase of Testes

Holding the 6 dpp testes at hypothermic temperature (~4°C) was performed as depicted in the experimental outline (**Figure 1**). Briefly, the excised 6 dpp testes were cultured directly or transferred to tubes containing α-MEM + Glutamax media supplemented with 10% knock-out serum replacement (KSR; 10828-010; Gibco™, Grand Island, USA), with Pen-Strep and Nys, using a sterile forceps. The tubes were placed in hypothermic, i.e., in a cooling unit maintained at ~4°C. The holding phase interval was scheduled as 6 and 24 h, corresponding to short-range

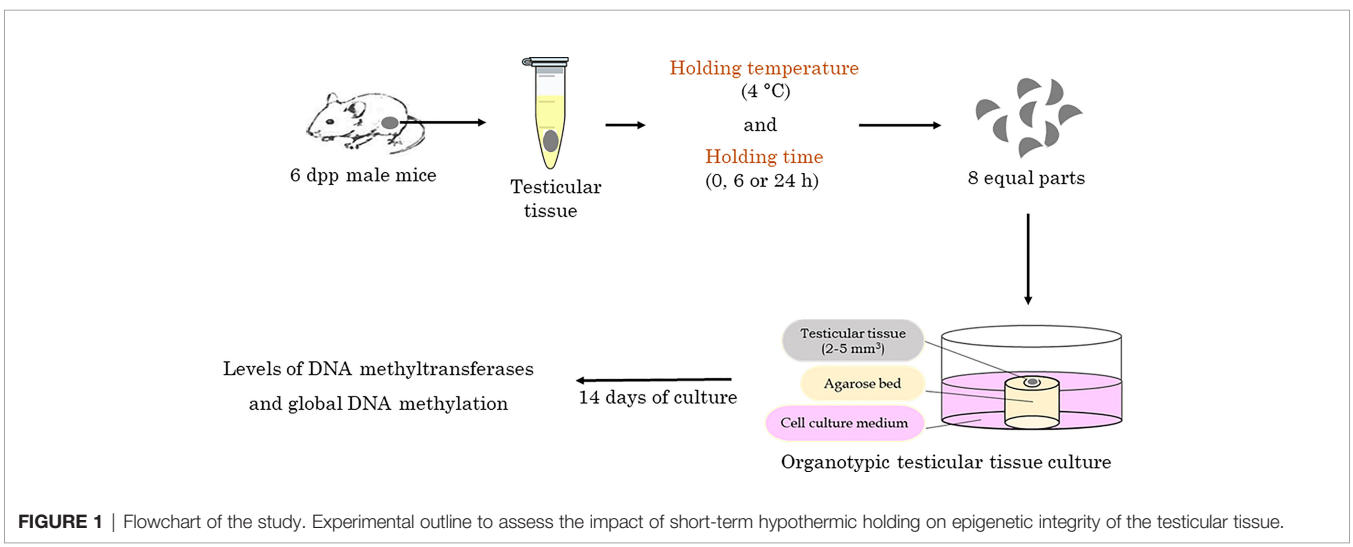

FIGURE 1 | Flowchart of the study. Experimental outline to assess the impact of short-term hypothermic holding on epigenetic integrity of the testicular tissue.

and long-range shipment. Post holding, these testes were processed for organotypic culture as described previously (12).

Isolation of Total RNA, cDNA Synthesis, and Gene Expression Analysis

Total RNA was extracted from ITT using TRIzol reagent (15596018, Ambion life technologies, USA). 1 µg of total RNA was reverse transcribed using random primers by a high-capacity cDNA RT kit (4368814, Applied biosystems, USA) according to the manufacturer`s protocol.

Quantitative polymerase chain reaction (*q*PCR) was carried out using Premix Ex Taq kit (RR390A, TaKaRa Bio, Japan), in StepOne™ Real-Time PCR System (Thermo Fisher Scientific, USA). TaqMan assay (Thermo fisher scientific, USA) for DNA methyltransferases viz. *Dnmt1* (Mm01151063_m), *Dnmt3a* (Mm00432881_m) and *Dnmt3b* (Mm01240113_m1) were used. *q*PCR results were normalized to *Actb* and *Gapdh* reference genes.

DNA Extraction and Global DNA Methylation Analysis

DNA was extracted from 25 mg of cultured ITT using QIAamp DNA Mini Kit (51306, Qiagen, CA, USA) according to the manufacturer`s protocol. Extracted DNA samples were eluted with 100 µl of TE buffer and stored at -20°C until further needed. The global DNA methylation was measured using MethylFlash™ Methylated DNA Kit (P-1034-96, Epigentek, NY, USA) according to the manufacturer's instructions. Briefly, methylated DNA was detected by 5-methyl cytosine (5-mC) antibody and quantified by colorimetric absorbance method at 450 nm using Multiskan™ FC Microplate Photometer (51119000, Thermo fisher scientific, Massachusetts, USA). The amount of methylated DNA was proportional to the OD intensity measured. Percent global DNA methylation (%5-mC) was calculated from the OD by the generated standard curve.

Statistical Analysis

Data were expressed as Mean ± SEM. The data were analyzed for normal distribution by the Shapiro-Wilk test. All the parameters were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 8 (GraphPad Prism software, CA, USA). The data were considered significant at $p < 0.05$.

RESULTS

Effect of Holding the ITT on mRNA Expression of DNA Methyltransferases

To assess the effect of ITT holding at 4°C for varying duration on the mRNA expression of DNA methyltransferases, *Dnmt1*, *Dnmt3a*, and *Dnmt3b* was analyzed using real-time *q*PCR. The relative expression of all three genes was normalized against the 6 dpp control group. The relative expression of maintenance DNA methyltransferases, *Dnmt1* was comparable in all the holding groups (**Figure 2A**). Further, the levels of mRNA transcripts of *de-novo* methyltransferases, *Dnmt3a* and *Dnmt3b* did not vary significantly in post-organotypic cultured ITT held at 4°C for various time periods (**Figures 2B, C**). Lack of statistical significance could be attributed to the variations in Ct values of real time PCR.

Effect of ITT Holding Prior to Organotypic Culture on Global DNA Methylation

5-methylcytosine (%5-mC) level was analyzed to explore the impact of holding temperature and length of holding on global DNA methylation. Though a moderate decline in %5-mC level was observed in 6 and 24 h holding time in comparison to 0 h, the differences were not statistically significant (**Figure 3**). This observation indicates that short-term hypothermic storage of ITT does not alter the global DNA methylation level post-organotypic culture.

DISCUSSION

The correct establishment of DNA methylation in developing germ cells depends on DNMT expression. The results from this study have demonstrated that short-term hypothermic holding

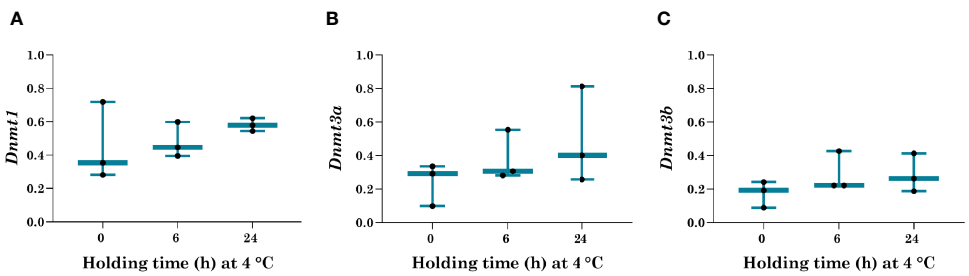

FIGURE 2 | mRNA expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b*. Real-time *q*PCR analysis to understand the effect of varying holding duration at 4°C on mRNA levels of **(A)** *Dnmt1*, **(B)** *Dnmt3a*, and **(C)** *Dnmt3b*. ITT held for 0 h at 4°C cultured for 14 days was used as a control in comparison to 6 and 24 h held cultured ITT in similar conditions. mRNA level of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* gene was normalized against reference genes *Actb* and *Gapdh*. Data are presented as Mean ± SEM (n = 3).

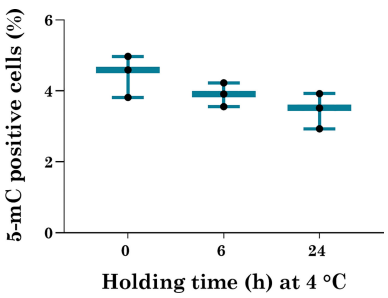

FIGURE 3 | Global DNA methylation analysis in cultured ITT. Levels of percent methylated cytosine (5-mC) in cultured ITT. Cultured ITT held at 0 h intervals were used as a control in comparison to cultured ITT held for 6 and 24 h at 4°C. The data is presented in mean ± SEM (n = 3).

of mouse ITT up to 24 h has no significant impact on the expression of DNMTs and global DNA methylation in organotypic cultured ITTs.

Previous studies have emphasized the importance of conventional DNA methylation during male germ cell development. DNA methyltransferases have dynamic expression during the proliferation and differentiation phase of spermatogenesis (24, 25). Targeted deletion of *de-novo* methyltransferases in prenatal male germ cells showed lower levels of DNA methylation in postnatal spermatogonia. Also, spermatogenic arrest and infertility were observed in such methyltransferases, deficient mouse models (24, 26, 27).

Therefore, in this study, we investigated the expression of DNMTs in cultured ITT after the tissue was subjected to hypothermic holding up to 24 h. The relative expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* did not vary significantly between the varying holding time periods tested. This observation is in agreement with previous reports where the expression patterns of DNMT's were unchanged in *in vitro* and *in vivo* derived spermatozoa (22, 23, 28). It has been also shown that fresh and frozen-thawed ITT can maintain DNMT1 and DNMT3A expression even up to 30 days of *in vitro* culture (23). Furthermore, spermatogonial stem cells obtained from non-human primates could maintain DNMT expression during short-term culture *in vitro* (22). Our data add new information to the existing literature that holding ITT up to 24 h at 4 °C has a minimal adverse effect on the DNA methylation process. Nevertheless, the Ct value variations in three trails could have affected the level of statistical significance. Hence, observations made in this study should be considered with caution.

The establishment of global DNA methylation in spermatogonial stem cells plays a key role in spermatogonial identity, its differentiation potential, and the accurate transmission of epigenetic information to the next generation (14, 15, 29). Most of the studies examining the global DNA methylation level in mouse testis were found to be stable at the postnatal period (29, 30). Also, Spermatozoa produced from fresh/cryopreserved *in-vitro* matured ITT had un-fragmented and condensed nuclear DNA (31).

Hence, it is important to understand the impact of ITT manipulation on the global DNA methylation level as fertility preservation techniques can coincide with the window of the establishment of global DNA methylation. Our observation showed hypothermic holding of ITT at 4°C for 24 h could decrease the global DNA methylation level (%5-mC) moderately, though it is not possible to establish the statistical significance in our study. Earlier, it has been shown that sperm derived from frozen-thawed ITT had a similar intensity of 5-mC compared to sperm derived *in vitro* (23).

While fully acknowledging the limitations of this approach in the mouse model, we feel that the results we presented in this report will be of significant interest to the field. We show that short-term holding of ITT at 4 °C does not affect the DNA methylation process. However, future research should focus on addressing the methylation errors in specific imprinted genes in human prepubertal tissues.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Kasturba Medical College & Kasturba Hospital Institutional Ethics Committee, approval #IAEC/KMC/93/2013.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: SA. Performed the experiments and was involved in the acquisition of data: RP and SRS. Analyzed and interpreted the data: RP and SU. Wrote the manuscript: SKA, SS, RP, and SU. Revised the manuscript critically for important intellectual content: GK and SG. RP is the guarantor of this work and as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have given final approval for publication.

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REVIEW
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Spermatogonial Stem Cell-Based Therapies: Taking Preclinical Research to the Next Level

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Fertility preservation *via* biobanking of testicular tissue retrieved from testicular biopsies is now generally recommended for boys who need to undergo gonadotoxic treatment prior to the onset of puberty, as a source of spermatogonial stem cells (SSCs). SSCs have the potential of forming spermatids and may be used for therapeutic fertility approaches later in life. Although in the past 30 years many milestones have been reached to work towards SSC-based fertility restoration therapies, including transplantation of SSCs, grafting of testicular tissue and various *in vitro* and *ex vivo* spermatogenesis approaches, unfortunately, all these fertility therapies are still in a preclinical phase and not yet available for patients who have become infertile because of their treatment during childhood. Therefore, it is now time to take the preclinical research towards SSC-based therapy to the next level to resolve major issues that impede clinical implementation. This review gives an outline of the state of the art of the effectiveness and safety of fertility preservation and SSC-based therapies and addresses the hurdles that need to be taken for optimal progression towards actual clinical implementation of safe and effective SSC-based fertility treatments in the near future.

Keywords: spermatogonial stem cells, fertility preservation, fertility restoration, childhood cancer, preclinical research, spermatogonial stem cell transplantation, testicular grafting, *in vitro* spermatogenesis

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INTRODUCTION

In recent decades, spermatogonial stem cell (SSC)-based therapeutic fertility approaches have become an important topic of investigation to overcome gonadotoxic treatment-induced infertility. The increasing survival rates over time of young cancer patients have highlighted the impact of gonadotoxic cancer treatments and the consequences for their fertility later in life. Currently, there is a survival rate of 80% among pediatric cancer patients in Europe (1), leading to a large population of childhood cancer survivors who are at risk of cancer treatment related infertility, for whom these SSC-based therapeutic approaches may be an opportunity to father genetically related children.

In the male, functional spermatogenesis is crucial for fertility. Spermatogenesis is initiated at puberty and relies on functional SSCs, which are located at the basement membrane of the seminiferous tubules

within the testes. The SSCs will either self-renew to maintain the number of stem cells or become differentiating spermatogonia that will develop into spermatocytes which, after two subsequent meiotic divisions, form haploid spermatids that will ultimately give rise to spermatozoa. Spermatogenesis is tightly regulated by the surrounding testicular somatic cells including Sertoli cells, peritubular cells and Leydig cells (2). Irradiation and the majority of chemotherapeutic agents use mechanisms that target proliferating cells which, unfortunately, include the proliferative SSCs, thereby impairing spermatogenesis (3). Prior to such a gonadotoxic treatment, adult men are given the option to cryopreserve sperm to enable them to sire a child *via* current fertility treatments. For young boys who have to undergo a gonadotoxic treatment, but do not produce sperm yet, there is currently no option to preserve and later on restore their fertility. However, the immature pre-pubertal testis does contain SSCs and cryopreservation of a testicular biopsy prior to the gonadotoxic treatment is offered in multiple medical centers around the world. Although still under development, SSC-based treatments thus provide an opportunity to preserve and restore fertility for pre-pubertal boys (4, 5). The development of methods for preservation and restoration of fertility has become an important aspect of research to further enhance the quality of life for male patients requiring gonadotoxic treatment during childhood. In trying to offer fertility preservation and restoration options, many avenues have been pursued through use of the SSCs residing within testicular tissues after birth (**Figure 1**). With the *in vivo* capacity of developing into sperm, SSCs are prime candidates for possible interventions to restore fertility, which include spermatogonial stem cell transplantation (SSCT), testicular tissue grafting and *in vitro* or *ex vivo* spermatogenesis.

In this review we will pinpoint which hurdles still need to be overcome in (preclinical) research to fulfill the promise of SSC-based approaches, in anticipation of providing these fertility treatments for patients.

TISSUE SOURCES FOR RESEARCH

The use of testicular tissue is a prerequisite for preclinical research on SSC-based techniques. In designing research and moving towards clinical applications, the target patient group should always be taken into consideration and the tissues used should represent this population as closely as possible.

In addition to a scarcity of available human testicular tissue for research in general, not all testicular tissues are equally suited for all lines of research in SSC-based approaches, as inherent differences can affect the presence and functionality of specific cell populations present within the testicular tissue that might influence the success rate of SSC-based research (**Table 1**). This is illustrated by the fact that prenatal, neonatal and adult tissues show distinct germ cell populations (6) and even within prenatal tissues differences in spermatogonial populations are evident between the sequential trimesters (7, 8). In addition, testicular somatic cells gain maturity over time, thereby altering the testicular micro-environment in the tissues used for research.

For preclinical research on SSC-based therapies there is a clear preference for the use of pre-pubertal tissue, both because of good representation of the intended patient population as well as the technical suitability of this tissue for these studies.

When pre-pubertal material is not available, research on SSCT can use adult testicular tissues, preferably of those

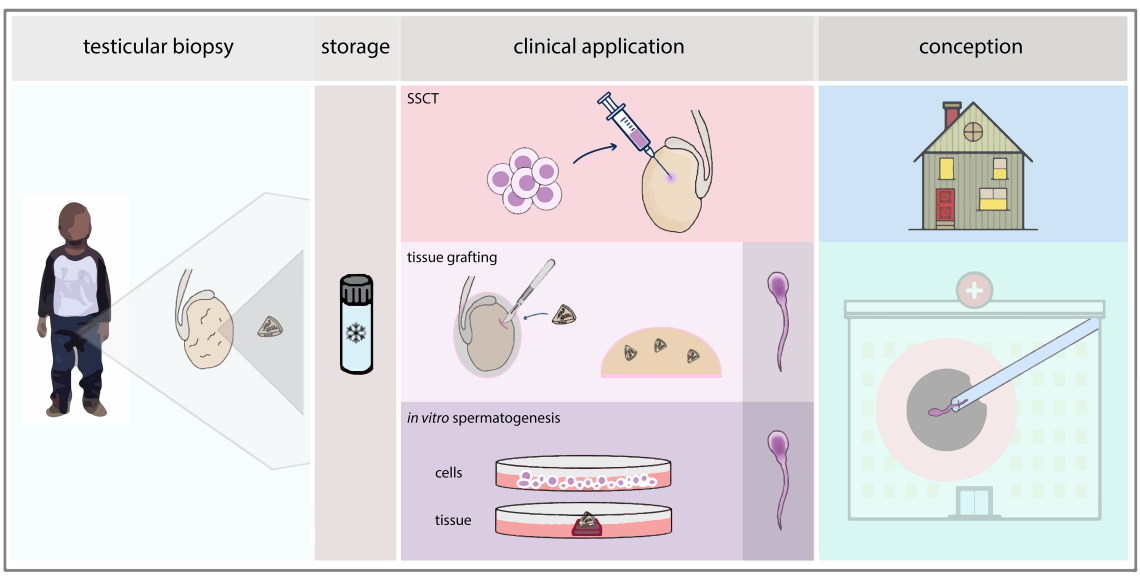

FIGURE 1 | Pathways in fertility restoration. After cryopreservation of a testicular biopsy, clinical application of SSC-based techniques can be sought *via* SSCT, testicular tissue grafting or *in vitro* spermatogenesis, either through cell-based or tissue-based culture. For SSCT, natural conception could be achieved whereas for therapies based on grafting or *in vitro* spermatogenesis, an ICSI procedure would be performed using elongated spermatids derived from the technique.

TABLE 1 | Suitability of various testicular tissues as sources for preclinical research on fertility restoration techniques.

Human tissue	SSCT	Grafting	*In vitro* spermatogenesis
Fetal tissue, no genetic abnormalities relating to reproduction	+-	+	+
Neonatal tissue (of child up to 1 month of age), no genetic abnormalities relating to reproduction	+-	+	+
Pre-pubertal tissue, no genetic abnormalities relating to reproduction	+	+	+
Adult tissue, normospermia (e.g. retrieved from orchiectomy in treatment of prostate cancer)	+	-	-
Adult tissue, influenced by hormonal treatment prior to sex reassignment surgery	+	+*	+*
Adult tissue, obstructive azoospermia	+	-	-
Adult tissue, non-obstructive azoospermia: maturation arrest	+-	-	-
Adult tissue, non-obstructive azoospermia: SCO-syndrome	-	-	-
Adult tissue, Klinefelter syndrome or likewise genetic abnormalities	-	-	-
Adult tissue, testicular cancer	-	-	-
Adult tissue, epididymal disorders	+	-	-

*+ appropriate source for preclinical research; +- use with caution; - advised not to use for preclinical research; *suitable when spermatogenesis is reduced to spermatogonia only. To distinguish between the different SSC- based techniques, shading correlates to the colors in the figure.*

patients in which the functionality of the SSCs has been established through histological detection of complete spermatogenesis. Tissues bearing pathologies that affect the spermatogonial population, such as congenital abnormalities or acquired Sertoli cell only (SCO) syndrome, are unsuitable for *in vitro* studies on SSC proliferation because of the absence of (functional) SSCs. Pathologies which only affect germ cells during their post-spermatogonial differentiating stages, as is the case in non-obstructive azoospermia patients with maturation arrest at the level of meiosis or spermiogenesis, may leave spermatogonial function unaffected. However, the level of maturation arrest may influence culture outcomes when propagating SSCs, as *in vitro* culture of testicular cells with early (pre-meiotic) maturation arrest may lead to more colonies, with larger diameters, than cultures using testicular cells of tissues with late (post-meiotic) maturation arrest (9).

For research on testicular tissue grafting and *in vitro* spermatogenesis, adult testicular material is unsuitable as study results would be influenced by either the presence of already ongoing spermatogenesis or, in its absence, by factors such as maturation arrest hampering any possibility of differentiation. The use of mouse neonatal or pre-pubertal testicular tissue for grafting in mice has been successful in the derivation of fertilization-capable sperm from the graft (10). However, caution is warranted with optimization of grafting protocols using human neonatal or fetal tissues, as the immature population of germ and somatic cells may respond differently than the germ cells present within the tissue of the pre-pubertal patient population.

Generally, testicular tissues derived from patients with underlying conditions, or undergoing treatments that affect meiosis and/or spermiogenesis, such as hormonal treatments for gender dysphoria (11, 12) or infectious diseases (13), could be used under the assumption that spermatogonial function is not affected. It is known that the vast majority of testicular tissue obtained during orchiectomy in transwomen contains spermatogonia (14). However, to our knowledge, the effects of these conditions on SSCs have not been systematically studied.

Likewise, when a patient has already started chemotherapy or radiation regiments, the SSCs within their testicular tissue may have deteriorated or undergone changes that will cause impairment or apoptosis of SSCs *in vitro* (15–17).

Finally, the use of tissues containing testicular cancer is not recommended for any preclinical SSC-based research as cellular function may be influenced by the cancerous cells. In the case of germ cell neoplasia *in situ* (GCNIS) these cancerous cells, which originated from precursors of SSCs, will have many molecular characteristics similar to SSCs, which may influence study outcomes (18).

To interpret results in preclinical research on SSC-based approaches, thorough documentation of patient characteristics, demography and (testicular) pathology of tissues used in these studies is essential.

CRYOPRESERVATION

For the future clinical application of SSC-based therapies, the testicular tissue biopsies are cryopreserved until the patient has a wish to have children, years after the pre-pubertal tissue biopsies were taken. During the freezing process of this testicular tissue, testicular cells might suffer from cryogenic damage caused by the formation of ice crystals. In an isotonic solution, water will crystallize spontaneously at -10°C (19). As a result, the osmolarity of the solution will increase, which will cause the cells in solution to lose intracellular water. When the cooling rate is too high, the cells are not able to lose all the intracellular water in time and intracellular ice crystals will be formed. When the cooling rate is too low, the cells will be exposed to a hyper-osmotic environment for too long, which will cause cell shrinking and apoptosis. To prevent these forms of cryodamage, different freezing methods, devices and cryoprotectants can be used for correct storage of biopsies, to allow future use of the tissue in SSC-based therapies (19).

The freezing of either a testicular cell suspension or testicular tissue have been proposed as options to cryopreserve SSCs. The freezing of testicular tissue has preference due to greater viability of germ cells and in particular SSCs, compared to the freezing of a testicular cell suspension (20). Furthermore, the cryopreservation of intact testicular tissue fragments permits the future opportunity to pursue tissue-based therapies as well as retaining the option to generate a cell suspension from the testicular fragments for stem cell-based therapies. For cryopreservation, the testicular

tissue is divided into small fragments (approx. 3 mm^3), which are deposited in cryotubes or cryostraws containing a cryoprotective medium.

Currently, no standardized cryopreservation method has been established that is optimal for male fertility preservation. However, the most commonly used freezing protocol is controlled slow freezing (CSF) with seeding. In this method, testicular fragments in 5% dimethylsulfoxide (DMSO) are cooled with a rate of 1°C/min, maintained at 0°C for 5 min, followed by further cooling at 0.5°C/min until −8°C for hand seeding and continued freezing until −40°C and subsequently, after a short stop, to −70°C with a cooling rate of 7°C/min before transfer to liquid or vaporized nitrogen (21).

An alternative to slow freezing is the use of solid-surface vitrification (SSV). Using this method, a droplet of DMSO-based cryoprotectant containing tissue fragments is exposed to liquid nitrogen and subsequently stored (22). However, slow freezing seems to provide greater protection to testicular tissue than SSV. This is in line with other studies reporting the ability of a slow freezing protocol to maintain tubular architecture and integrity of pre-pubertal human testicular tissue fragments (21, 23–31).

Besides the different freezing regimes, various cryoprotectant freezing media can be used. To examine the effect of cryopreservation on testicular tissue fragments, Keros and colleagues analyzed morphology and structure of the tissue as well as the presence of MAGE-A4+ spermatogonia using light- and electron microscopy (21). In these experiments, the fragments were thawed and subsequently cultured for one day at 33°C in 5% CO_2 prior to fixation. The results indicate that a freezing medium containing 5% DMSO (0.7 mol/l) during cryopreservation causes a reduced amount of cryodamage in comparison to other cryoprotectants such as 1.5 mol/l 1,2-propanediol (PrOH). Over 90% of MAGE-A4+ cells were undamaged (i.e. not detached from the basement membrane or neighboring Sertoli cells) when using DMSO, compared to 60% using PrOH.

This is in agreement with results showing that cryopreservation of testicular tissue by slow-freezing in 5% DMSO does not seem to affect the long-term culture of human testicular cells with regard to the presence of germ cell and somatic cell populations (32). Further optimization and possible addition of novel cryoprotectants, such as the antioxidant pentoxifylline (33) to the freezing medium may further improve the preservation of human SSC viability. Our research group cryopreserved testicular tissues obtained from orchiectomies of two adult patients with prostate cancer using various protocols in a pilot study. We compared cryoprotectant TEST-yolk buffer (TYB) with 7.5% human serum albumin (HSA, Albuman), 5% DMSO with 5% HSA and 8% DMSO with 20% HSA using a controlled slow freezing method described for tissue CSF as described above (21). Furthermore, we tested other freezing methods by bringing the straws with tissue fragments and cryoprotectant directly in the nitrogen vapor or using a shorter method of controlled slow freezing originally designed for cryopreserving sperm (CSFS), during which cryostraws were cooled with 0.5°C/min to 5°C followed by a cooling rate of 2°C/min until the samples reached 2°C. Finally, the samples were cooled until -80°C with a cooling rate of 10°C/min. No seeding was performed in this method. Morphology of the tissue was assessed after a 24-hour culture compared to fresh tissue by MAGE-A4 staining. Long-term cultures of isolated testicular cells from these tissues were assessed as well, including by SSC colony counts. Morphological damage was most pronounced in all CSFS frozen tissue and was also found to be increased in all those testis fragments cryopreserved in TYB-containing medium compared to align with DMSO-containing medium. The immunostaining of MAGE-A4+ cells in these tissues, 24 hours after culture, reflected these results. When testicular cells were isolated and cultured, cells frozen by CSFS and in nitrogen vapor of one patient did not survive long term culture. However, the cultures of isolated cells from testicular fragment frozen tissue in all other conditions were all capable of SSC colony formation, indicating presence and proliferation of SSCs. Collectively, the slow freezing method CSF (21) with 5% or 8% DMSO as a cryoprotective agent appears to be favorable for cryopreserving testicular tissue for future *in vitro* spermatogonial proliferation prior to transplantation as a fertility restoration treatment.

The fertility restoring potential of cryopreserved tissue has often been investigated with regards to testicular grafting, with the use of both fresh (10, 34, 35) and cryopreserved tissues (36–40) which have been shown to be successful in pig, mice and primate models. Direct comparisons of the use of fresh versus frozen-thawed tissues intended for tissue grafting have been performed in various species, but show variable results. In mice, spermatogenic progress within the testicular fragments after grafting was similar between grafted fresh tissue fragments and fragments that were frozen-thawed prior to grafting (41). In a later study, a lower number of intact tubuli at one day after grafting within grafted frozen-thawed tissues was found, but at later timepoints, up until two months, no differences were observed between grafted fresh and frozen-thawed tissues (42). Fayomi et al. did not show any differences between fresh and frozen-thawed conditions regarding graft weight, stage of spermatogenesis or percentage of tubuli with complete spermatogenesis, whether the grafts were ectopically or orthotopically placed (43). The use of human frozen-thawed testicular tissues xenografted in mice resulted in similar levels of structural integrity and germ cell survival as the use of fresh tissues, although the number of Sertoli cell-only (SCO) tubules was higher in the frozen-thawed tissue group (44).

For *in vitro* spermatogenesis with testicular tissue, cryopreserved testicular tissues have also been tested compared to fresh testicular organ culture. The organ culture used was first described for mouse testis fragments (45). This group compared fresh with cryopreserved mouse testis tissue with regard to their ability to develop spermatogonia to germ cells of late meiosis onwards under the reporter of *Gsg*-GFP (46). They applied uncontrolled slow freezing, using a Bicell biofreezing vessel, with a cooling rate of 1°C/min until -80°C before plunging in liquid nitrogen, using either 1.5 mol/l (11%) DMSO, 1.5 mol/l PrOH or Cell Banker 1 as cryoprotectant, or vitrification with Stem Cell Keep as cryoprotectant. They found that slow freezing with DMSO and vitrification with Stem Cell Keep showed similar progression of

spermatogenesis as fresh cultured tissue, based on GFP expression levels. Also, for human testicular organ culture the use of fresh and cryopreserved tissues using CSF with 5% DMSO were compared (47). Reassuringly, no significant difference in culture outcome on spermatogonial maintenance and proliferation and hormone production between fresh and cryopreserved testicular fragments of human pre-pubertal boys could be found (47).

SSC-BASED TECHNIQUES

In Vitro Proliferation and Transplantation of SSCs

Auto-transplantation of SSCs harvested from the patient prior to treatment could provide recolonization of the seminiferous tubules and subsequent *in vivo* spermatogenesis, in theory restoring fertility and the ability of the patient to achieve progeny through natural conception (**Figure 1**). To reintroduce the SSCs into the human testis, the preferential method seems the ultrasonically-guided injection of cells in the rete testis, a comprehensive review of which is written by Gul et al. (48).

Brinster et al. were the first to demonstrate the process of SSCT to be successful in establishing colonization and spermatogenesis in recipient testes in mice (49, 50), as well as in resulting in offspring with the donor haplotype (50). Since then, auto- or allo-transplantation of SSCs has been achieved in various mammals, including rodent and non-rodent species, as reviewed by Takashima & Shinohara (51), and non-human primates (52, 53), resulting in functional spermatogenesis within the recipient animals.

In humans, clinical trials with SSCT have not yet been established; a single report on SSCT in seven men receiving injections of cryopreserved testicular cells has not been followed up by a report on the outcome of these procedures (54). However, from studies in mice it has become clear that transplanted numbers of SSC colonies gradually decrease during the homing process after transplantation (55) and that the concentration of transplanted SSCs is highly linked to the success of colonization of SSCs (56) and donor-derived spermatogenesis within the recipient testis (57).

Because only biopsies of limited size can be collected from patients, propagation of SSCs retrieved from these biopsies will be necessary to achieve sufficient numbers for successful transplantation and recolonization of the seminiferous tubules within the recipient testis. Kanatsu-Shinohara et al. first demonstrated effective long-term propagation of murine SSCs, which were able to restore fertility in sterile recipients (58). In humans, long-term propagation of testicular cells was demonstrated to be possible as well, with cultured testicular cells from both adult (59) and pre-pubertal tissues (60). Identity and propagation between two time points in culture of these human SSCs could be demonstrated through xenotransplantation into sterile mice, by counting donor SSCs that had migrated to the spermatogonial stem cell niche within the seminiferous tubules.

Still, the identification of SSCs within a testicular tissue or cell culture *in vitro* is a major challenge. Testicular cell cultures include both somatic cells and germ cells, the latter of which is a heterogeneous group of spermatogonia of varying states of differentiation. At this moment there is no agreement on a specific, unambiguous marker for human and/or non-human primate SSCs *in vivo* and *in vitro*. For many currently used markers, including ITGA6, KIT, GPR125 and DAZL, expression is not strictly limited to spermatogonia and can also be found in testicular somatic cells (61, 62). For other markers, spermatogonial expression of the marker is controversial, as not all study results are in agreement. This is the case for GFRA1, THY1 and UCHL1 (22, 61, 63–79). Expression of certain markers can also rely on the developmental stage of the testicular tissue; for instance, expression of POU5F1 is limited to subpopulations of fetal and neonatal germ cells (gonocytes) in humans and expression is downregulated along with progressing differentiation (7, 8, 18, 80).

Single-cell sequencing studies have identified various 'states' of germ cells, with progression of development of differentiation from State 0 to State 4, where each state is characterized by a unique set of markers, although at times partially overlapping (6, 81). UTF1 and PIWIL4 are examples of markers expressed in the earliest state, with PIWIL4 being the more specific marker (6, 82, 83). Another newly identified candidate marker for undifferentiated spermatogonia, carrying SSC characteristics, involves the LPPR3 protein (6).

Furthermore, it is uncertain whether various testicular cell types retain their transcriptomic and metabolic signatures when isolated from their natural niche (62). Due to these dynamic processes, the heterogeneity of the testicular cell populations and the many potential markers to choose from, it is difficult to establish inter-study comparisons of culture outcomes and efficiency with regard to SSC propagation. To extend studies into the field of SSCs and their potential use, the scientific community would benefit from more insight into specific markers for clearly-identified spermatogonial populations, combined with functional studies as to their SSC potential with regard to *in vitro* use. In identifying markers, a focus on surface markers as opposed to nuclear markers may be more beneficial for isolation and enrichment of SSCs from cryopreserved biopsies or after culture.

Currently, the gold standard to demonstrate SSC functionality is still the (xeno)transplantation assay, in which donor-derived SSCs are shown to be capable to migrate to the stem cell niche within the seminiferous tubules of the recipient testis and, in a compatible species, initiate and maintain donor-derived spermatogenesis, although this latter part cannot be demonstrated in human to mice xenotransplantation assays.

In working towards clinical application of SSCT, study protocols have to be adapted to clinical requirements and regulations. Therefore, it will be essential to work towards xenofree, clinical grade media and methods in compliance with good manufacturing practice (GMP) and good clinical practice (GCP) systems, while adhering to the regulations regarding the production of advanced therapy medicinal products (ATMP, regulation no. 1394/2007 [European Medicines Agency (EMA)] (84).

The culture methods and media should support SSC proliferation while preventing their differentiation. Besides basic nutrients, additional components such as specific cytokines, metabolites, hormones and other signaling molecules, that are known to stimulate spermatogonial proliferation, may be included in the culture medium. In 2003, Kanatsu-Shinohara et al. developed a successful culture protocol, using medium based on StemPro-34 Serum Free medium, for long-term *in vitro* propagation of murine SSCs (58). Although a comparable medium can also support long-term propagation of human SSCs (59, 60), overgrowth of testicular somatic cells within the culture system remains problematic, as the SSC signature is diluted over time (62). The presence of feeder cells is beneficial to the survival of SSCs as they provide mechanical and metabolic support and paracrine signals (85, 86). The use of exogenous feeder layers however is unsuitable for clinical implementation of the technique. Therefore, somatic cells which support SSC proliferation and which are already present within the testicular suspension, isolated from a testicular biopsy, are used for SSC culture. To prevent overgrowth of these somatic cells, the culture method and medium used should steer towards an optimal balance between the growth of somatic cells and SSCs. Alternatively, the testicular somatic cells may be replaced by a (synthetic) matrix for cell support, combined with supplementation of the medium with nutrients and growth factors to ensure the metabolic support for SSCs otherwise provided by the feeder cells. This process however heavily relies on the ability to isolate and remove the somatic cell population from the germ cell population prior to culture, which is currently limited by the lack of a specific SSC marker (87, 88).

Optimization of the method for *in vitro* culture of human SSCs is thus a necessary step prior to clinical implementation. Investigation of the SSC niche in humans might be a pivotal step in identifying the factors necessary for effective maintenance and propagation of SSCs (89).

Grafting of Testicular Tissue

In vivo spermatogenesis can also be achieved through autologous grafting of immature testicular tissue fragments underneath the skin of the patient (**Figure 1**). Through angiogenesis, the tissue receives endocrine signals from the body circulation which enable spermatogenesis within the tissue (90, 91). After full progression of spermatogenesis, spermatids can then be harvested from the retrieved graft and used in current assisted reproductive techniques like intracytoplasmic sperm injection (ICSI).

Testicular (xeno)grafting has been shown to lead to successful spermatogenesis in testicular tissues of many mammalian species, including mice, pigs and monkeys (34, 35, 37), subsequently leading in some cases to live offspring through application of ICSI (10, 35, 40, 43, 92). Host conditions may impede full spermatogenesis within xenotransplants with cryopreserved pre-pubertal tissues (39), although in non-human primates complete spermatogenesis has been achieved with autologous transplantations of fresh testicular pre-pubertal tissue as well as with xenografts of similar origin (34, 93).

In the xenografting of non-human primate testicular tissue to mice, the spermatogenic maturation depended on the location of the graft, with orthotopic (intra-testicular) grafts leading to further spermatogenic progression in a higher number of grafts than ectopic grafts under the skin of the back (38, 94). The ectopic location of the grafts in these experiments may have caused a higher tissue temperature and different hormone concentrations than those physiologically present in the scrotal area, potentially contributing to spermatogenic arrest. Recently, Fayomi et al. succeeded in fertilization of primate oocytes using autologous graft-derived primate spermatozoa, resulting in the birth of a healthy rhesus macaque (43). As of yet, no autologous grafting has been described with human tissue; only xenografts to mice have been studied. The use of human tissue in xenografts to mice has not yet resulted in complete spermatogenesis (95, 96), although meiotic activity could be observed (44).

For clinical use, the quantity of collected material is likely small, which could represent an obstacle for the success of this technique. Low survival rates of grafted tissues have been reported, potentially necessitating unilateral orchiectomy to retrieve sufficient amounts of material (39).

Success of this technique also relies on adequate vascularization of the grafted tissue, delivering both the necessary oxygen and hormones for SSC proliferation and differentiation. Hypoxia in the graft center may lead to loss of testicular tissue and all germ cells within (42). Vascularization of human grafts has been shown to benefit from a short-term culture supplemented with vascular endothelial growth factor (VEGF) prior to placement of the graft (93), but additional growth factors other than VEGF may be necessary for successful graft survival.

In Vitro or *Ex Vivo* Spermatogenesis

Spermatogenesis is one of the most complicated developmental processes in the human body, and thus remains very challenging to re-create by *in vitro* differentiation of stem cells or *ex vivo* cultures of testicular fragments (**Figure 1**). Nevertheless, application of *in vitro* or *ex vivo* spermatogenesis holds great potential for future fertility restoration or preservation, as haploid spermatids derived from these methods could be used for assisted reproductive technologies such as ICSI.

The use of SSCs as basis for *in vitro* spermatogenesis (97–101), isolated from a testicular biopsy taken prior to gonadotoxic treatment, would have high clinical relevance. Although many efforts have been made to re-create spermatogenesis *in vitro* using pluripotent stem cells (PSCs) (102, 103), blastocyst embryos to isolate the required embryonic stem cells (ESCs) will likely not be available for clinical use. Moreover, even though induced pluripotent stem cells (iPSCs), generated by genetic reprogramming of cells from the patient's own somatic tissues could be a possible alternative source, the safety of the use of iPSCs for reproductive purposes has still not been sufficiently investigated. However, regardless of PSCs or SSCs as the cell type of origin, the most challenging spermatogenic process to mimic *in vitro* is the process of meiosis, the process by which genetically different haploid spermatids are produced *via* two successive meiotic cell divisions. Although several studies reported generation of round spermatid-like cells *in vitro* using SSCs

(97, 98, 100, 101), only a few studies (98, 101) investigated the key meiotic events that are required for successful meiosis and true *in vitro*-derived gametes (104). When mouse SSCs were induced to complete meiosis, using immortalized Sertoli cells as feeder layer in a three-step induced culture system, despite the *in vitro* formation of pachytene-like spermatocytes, the homologous chromosomes did not display full synapsis and no meiotic crossovers were formed. Despite these meiotic problems, many cells still proceeded to the first meiotic division (MI), occasionally even forming round spermatid-like cells (98). Apparently, these aberrant spermatocytes were not timely eliminated by the meiotic checkpoints that normally (*in vivo*) induce apoptosis in order to prevent possible generation of aneuploid sperm (105). Because of a lack of meiotic crossovers in the *in vitro*-generated pachytene spermatocytes, the MI-spermatocytes did not form chiasmata (physical connection between the homologous chromosomes) and thus displayed univalent (pairs of sister chromatids) instead of bivalent (pairs of homologous chromosomes) chromosome pairs, which will almost certainly cause subsequent formation of aneuploid spermatids. Meanwhile, Sun et al. reported complete chromosome synapsis and meiotic crossover formation *in vitro* using human male germline stem cells (101). However, the detection of chiasmata in MI-spermatocytes was not described.

The difficulty in mimicking spermatogenesis *in vitro* could be due to the lack of a suitable testicular micro-environment needed to successfully support the spermatogenic process. Spermatogenesis *in vivo* requires spatio-temporal interactions between germ cells and testicular somatic cells. Therefore, including an *in vitro* somatic niche in culture could be instrumental in supporting the germ cells. In mice and humans several essential regulators, such as retinoic acid and gonadotropins, have been identified and are appropriately discussed in reviews such as that of Rombaut and colleagues (106).

Testicular somatic cells, including peritubular myoid cells, Leydig cells, Sertoli cells and endothelial cells, as well as immune cells, play important roles in supporting spermatogenesis. Unlike *in vitro* spermatogenesis, in *ex vivo* spermatogenesis methods where testicular fragments are cultured instead of isolated cells, testicular organization is still in place during culture, thereby circumventing the need to structurally re-create the right microenvironment.

The first successful *ex vivo* spermatogenesis was demonstrated in 2011 (45, 107). In this study, murine testicular fragments were cultured at a gas-liquid interphase using an agarose stand and *ex vivo* elongated spermatids were identified after five weeks of tissue culture and could be used for the generation of live offspring. Repeating this method for rat immature testicular tissue resulted in progression of spermatogenesis up to round spermatids (108). Studies using human testicular tissue for *ex vivo* spermatogenesis showed no initiation of meiosis (109, 110). Using histological analysis, one study reported that spermatids could be observed morphologically in *ex vivo* cultures with human testis tissue (111). No studies using human or non-human primate tissues describe the production of spermatids that were shown to be capable of fertilization.

Despite the achievements that have been reached with *ex vivo* spermatogenesis using tissue culture, the efficiency to perform *ex vivo* spermatogenesis is very low. More research is required to improve the efficiency and further translation of the method for primate testicular tissue.

More fundamental knowledge on non-human primate and human spermatogenesis and the differences with that of rodents will be helpful to successfully mimic this process through either *in vitro* or *ex vivo* spermatogenesis.

SAFETY

Biopsies for Fertility Preservation

As any medical procedure may involve short- or long-term health risks, it is important to consider the impact that taking testicular biopsies may have on the patient. In many cases, the patient's parent(s) or caretaker(s) will have to make the decision for the child to undergo this procedure and they will have to be duly informed of its potential risks, including those of the general anesthesia under which the procedure is performed. These risks however may be limited by concomitant execution of other medical procedures that are inherent to the initial (cancer) treatment, such as central venous line placement or bone marrow aspiration. Worries about the risks of the biopsy itself comprise the chance of acute post-operative complications and chronic changes within the testicular tissue. Post-operative complications include post-operative bleeding and wound infection. Rates of occurrence of these complications are low and vary between 0 – 3.8% (112–115). Transiently, extra- and intra-testicular hematomas may occur (112). Additionally, when the testicular biopsy is performed, to reduce the burden of repeated anesthesia, as an additional procedure during general anesthesia for a concomitant procedure, such as central venous line placement or bone marrow aspiration, this may lead to increased post-operative pain levels compared to the main procedure only, potentially necessitating additional analgesics in these patients (116).

Unilateral testicular biopsies of a maximum of 1 ml or 50% of total testicular volume of pre-pubertal patients did not lead to decreased growth, compared to the contralateral non-biopsied testis, as measured by ultrasound during one year after surgery (112). In 6.3% of the patients fibrotic testicular lesions were found using ultrasound as part of a long-term follow up (112). Although gonadotrophin levels have been observed to be abnormal in the follow-up of pre- and peri-pubertal boys from whom a testicular biopsy was taken, the incidence of these abnormal levels matches those of childhood cancer survivors who have not undergone testicular biopsies (113). Similarly, results of semen analyses of patients who had undergone testicular biopsies prior to treatment (88% of which with alkylating agents) showed comparable rates of normo-, oligo- and azoospermia to large-scale cohort studies of childhood cancer survivors (113).

In conclusion, the reproductive health of patients undergoing testicular biopsies does not seem to decline as a result of the testis biopsy procedure.

For fertility preservation, the biopsies are subsequently stored in nitrogen. Although cryopreserved tissue function has been shown to be similar to that of fresh tissue, as described above, the effect of cryopreservation on the genetic and epigenetic status of cells within cryopreserved tissues is largely unknown. Despite the availability of studies on molecular changes on cryopreserved sperm, oocytes and embryos (117), studies researching the effect of cryopreservation on SSCs are limited. In mice, SSCT was as successful with freshly isolated germ cells as with germ cells isolated from long-term cryopreserved tissues, in establishing spermatogenesis in recipient animals and subsequent fertilization of murine oocytes through ICSI (118). The resulting offspring did not show a significant amount of DNA copy number changes (chromosomal deletions, duplications) nor changes in DNA methylation patterns in whole-genome DNA analysis of liver tissue, compared to the offspring of wild-type mice, obtained through natural conception, suggesting that potential DNA methylation changes of SSCs in cryopreserved tissues are reversed upon transplantation, or in the offspring.

Safety of SSCT for Patients and Progeny

A major concern with regard to SSCT in cancer patients is the possible reintroduction of malignant cells from the original biopsy during the autotransplantation of (cultured) testicular cells to the patient who recovered from cancer. Research data indicate that acute lymphoblastic leukemia (ALL) cells do not survive for longer than 16 days in a human testicular cell culture system (119). *In vitro* propagation to increase SSC numbers prior to autotransplantation therefore seems to have the additional benefit of eliminating ALL-cells, although this may not be the case for other cancer types. Further purification methods, such as immunomagnetic bead-based sorting (MACS) using a combination of spermatogonial markers showed promising results in a mouse model (120). In contrast, the use of Percoll density gradients appeared insufficient to prevent transmission of leukemic cells to the recipient mice (120). Fluorescence-activated cell sorting (FACS) also showed promising results in experiments with human cells. The combined use of the markers EPCAM, HLA-ABC and CD49e allowed the separation of putative spermatogonia and leukemic cells prior to xeno transplantation. Tumor formation was only observed in the testis of transplanted animals having received the leukemic fraction, and tumors were absent in animals receiving the spermatogonia-enriched fraction, whilst the latter also showed the highest number of spermatogonial colonies (121). However, such separations are highly dependent on knowledge of specific antigens that are different between spermatogonial and cancerous cells. However, cancer cells are known to express many genes that are normally only present within germ cells (122, 123), including antigens that may be used for sorting (124). In addition, purification strategies need to be highly reliable, as only 20 leukemic cells are needed to cause a terminal relapse, as was shown in a rat model (125). Any patient-specific aberrations in tumor antigen expression could therefore be disastrous, when relying on the selection of cells by specific antigens. Prior to cell enrichment, a thorough characterization of the cancer cells from every individual patient would thus be pertinent.

Although a valid concern for cell culture and manipulation in general, the culture of human SSCs does not seem to induce chromosomal abnormalities (126, 127). This is similar to the genetic stability of long-term (over two years, with an approximate 10^{85}-fold expansion) cultured murine SSCs, which have been shown to maintain their androgenic imprint based on five imprinted regions and their capacity for spermatogenesis after transplantation, leading to fertile progeny (128). DNA methylation patterns of cultured human testicular cells, sorted for ITGA6, do not overlap with those found in seminomas (127), indicating cultured cells do not acquire a seminoma signature. However, specific DNA demethylation patterns of paternally imprinted genes and concurrent DNA hypermethylation of maternally imprinted genes were observed in cultured and enriched human spermatogonia (126). It is currently unknown whether these changes are correlated with the fertilization potential of spermatozoa that would be derived from these spermatogonia, or whether they pose a health risk towards the patient or their offspring, although studies in mice do not seem to indicate this (129, 130).

As there has not yet been a clinical trial investigating the application of SSCT in human patients, no information regarding the long-term health in such patients is available. However, research in mice shows no difference in life-span up to the age of 18 months between transplanted and non-transplanted groups of busulfan-treated animals, with no increase in the occurrence of malignancies (129). Regarding the offspring, in non-human primates the sperm derived from the donor-SSCs after non-cultured SSC allogenic transplantation has been shown to possess competence for fertilization *via* ICSI and subsequent embryonic development up to the morula stage (52, 53). However, (epi)genetic stability of these embryos was not assessed and no progeny was derived in this study, limiting assessment of safety in respect to health of the procedure in primates. Nevertheless, in mice the long-term follow up of two generations of progeny of transplant-recipient mice showed no significant differences compared to the control group regarding congenital abnormalities, childhood development, lifespan, reproductive health and adult general health (130). These findings are supported by studies in multiple mouse strains reporting no chromosomal abnormalities or genetic deviations (131). To add, no changes were seen in DNA methylation patterns in spermatozoa derived from animals receiving SSCT of non-cryopreserved and uncultured spermatogonia, nor in spermatozoa and somatic cells of multiple organs of their first and second generation offspring (132). However, a later study on transplantation of freshly isolated spermatogonia did find that the expression pattern of histone lysines H4K5ac and H4K8ac in murine germ cells in the various stages of the seminiferous cycle in testicular tissue of animals having received SSCT differed from that in controls, as analyzed by immunohistochemistry. However, H3K4me3, H3K9ac, H4K12ac and H4K16ac of germ cells after SSCT were comparable to controls, as was their DNA methylation status (133).

Safety of Graft Placement

As clinical trials have not commenced, no safety studies concerning human testicular grafts have been performed. While the main focus of studies has been to achieve

spermatogenesis within the graft, little has been published regarding the (epi)genetic stability of the procedure and the cells within the graft, for both the recipient and his progeny. In mice, graft-derived spermatids showed normal fertilization capacity and progeny derived from graft-derived sperm showed good reproductive health, as demonstrated through mating experiments (10). However, general health of this offspring was not assessed.

Studies in testicular grafts of rhesus macaques showed good fertilization potential of graft-derived spermatids through testicular sperm extraction and intracytoplasmic sperm injection (TESE-ICSI), with good embryonic development up to blastocyst stage (34) and even a healthy live birth of one female (43). Behavior assessments of this graft-derived infant were age-appropriate and no health defects were reported by the authors (43).

Although these results are promising, the basis of the presumed safety of this procedure is still very small. Using immunohistochemistry markers on mouse tissue, it was shown that, compared to wildtype animals, testicular grafting does not alter the spermatogenic methylation patterns of germ cells in various stages of the seminiferous cycle as analyzed by immunohistochemistry analysis of expression of DNA methyltransferases -1 and -3A and 5-methylcytosine (DNMT1, DNMT3A and 5-MC) (133). Concerning histone modifications on H3K4me3, H3K9ac, H4K5ac, H4K8ac, H4K12ac and H4K16ac, only H4K5ac showed a significant difference in expression pattern between grafted and control groups (133).

To our knowledge, there are currently no other studies on the genetic integrity of spermatids derived from (xeno)grafts. Similarly, reports on assessment of the health of the recipient and their progeny are limited. Furthermore, in anticipation of the clinical use of this technique, it will be essential to ascertain whether the placement of an autologous graft from a cancer patient with potential infiltration of systemic cancer in the testis will pose a risk of recurrence of the cancer after grafting. Further studies are needed to establish reliable methods to secure transplantations of cancer-free materials only (5).

Safety of *In Vitro* or *Ex Vivo* Spermatogenesis

Aneuploidy and Genomic Instability

For *in vitro* or *ex vivo* spermatogenesis, one of the foremost risks is the derivation of sperm with an incorrect number of chromosomes, referred to as aneuploidy. An estimated 20–40% of all human conceptions contains aneuploid cells, of which most are due to errors in meiosis (134). In all males, a proportion of sperm can be observed to be aneuploid (135, 136). Approximately 3–5% of spermatozoa of males with proven fertility are aneuploid, and these levels are significantly higher in infertile men (136–139). Aneuploidy in gametes may cause early pregnancy loss or severe developmental defects (140, 141). Meiotic problems, like incomplete chromosome synapsis and impaired recombination, are considered to be main factors in the emergence of chromosome nondisjunction and subsequent aneuploidy (138, 142, 143). In order to prevent generation of aneuploid sperm, key meiotic events such as chromosome synapsis, recombination and subsequent meiotic crossover formation, are strictly monitored by checkpoint mechanisms to timely arrest meiotic progression (105, 144). However, when spermatogenesis was re-created *in vitro* using mouse SSCs, these checkpoint mechanisms did not appear to be functional (98). For *ex vivo* cultures of testicular fragments, most meiotic events or meiotic checkpoint function have not yet been systemically investigated. Since meiotic DNA damage repair, recombination and checkpoints appear not to be fully functional in current *in vitro* spermatogenesis protocols, these processes should be thoroughly investigated and monitored before considering clinical application of *in vitro* or *ex vivo* spermatogenesis.

Epigenetics

Epigenetic modifications, being DNA methylation, histone modification and the production of small non-coding RNAs, regulate many processes of spermatogenesis. Besides spermiogenesis, also spermatogonial differentiation steps and early meiosis are subject to epigenetic regulation. For example, some changes in DNA methylation may occur in spermatogonia and early spermatocytes (145). These processes include meiotic silencing of unsynapsed chromosomes (MSUC), XY-body formation and the histone-to-protamine transition during spermiogenesis (146). These are all crucial for germ cell function and post-fertilization embryonic development (147). The establishment of epigenetic patterns during human germ cell development is dynamic and age and phase-dependent (148, 149). Aberrant DNA methylation during spermatogenesis may impair spermatogenesis, causing infertility (150). Despite post-fertilization (embryonic) epigenetic reprogramming as a mean to prevent aberrant progeny, successful fertilization of oocytes with epigenetically abnormal sperm may still pose a risk, because some abnormal epigenetic marks may persist (151) and potentially influence embryonic development and health of the offspring. In couples experiencing recurrent pregnancy loss, sperm showed more DNA methylation abnormalities of multiple imprinted genes, compared to couples without history of miscarriage or infertility (152), illustrating the potential detrimental effect of epigenetic aberrations in germ cell function.

Moreover, long-term culture of primary cells in general may induce changes in DNA methylation (153). Therefore, when spermatogenesis is re-created *in vitro*, the epigenetic status has often been investigated to make sure that no aberrant epigenetic patterns are present in the *in vitro*-derived germ cells (100, 101).

When murine SSCs were used as a starting point to generate a multipotent adult GSC line (maGSCs) that could subsequently be induced to differentiate into haploid cells, these haploid cells showed incomplete epigenetic imprinting of the H19 gene (100). Nevertheless, a study using human male germline stem cells reported normal epigenetic status in round spermatids-like cells (101).

The research on epigenetic status of cells derived from testicular tissue cultures is limited. The short-term culture of rat fetal testicular tissue shows a similar chronology in epigenetic remodeling of DNA methylation patterns in gonocytes, compared to gonocytes *in vivo* (154). Similarly, Yokonishi et al. described a normal state of methylation in the offspring

derived from the culture of cryopreserved mouse testicular tissue fragments (46). However, DNA methylation is not the only type of epigenetic regulation and more studies are needed to ascertain the epigenetic properties of cultured SSCs, spermatocytes and spermatids.

Limitations and Potential Risks of ROSI

Unlike *ex vivo* spermatogenesis, in which formation of elongated spermatids could be achieved that could be used for ICSI (45, 46, 155), differentiation of human SSCs by cell culture has so far, to our knowledge, only produced round spermatid-like cells (97, 98, 100, 101). Round spermatid injection (ROSI) technique would enable the use of these immature haploid precursors of spermatozoa to fertilize oocytes and give rise to the offspring. However, some concerns regarding ROSI have arisen as round spermatids and elongated spermatids have significant differences in chromatin structure (156). The use of round spermatids may not establish proper imprints or global methylation, thereby affecting embryonic development (157). Although in two human studies the children born after ROSI appeared healthy without any epigenetic problems (158, 159), data from a mouse study described that, in contrast of the normal paternal genome derived from mature spermatozoa, embryos derived from round spermatids appeared to have genome-wide aberrant DNA methylation of their paternal genome (160). This likely explains the observed poor development of these embryos. In addition, the overall low success rates of ROSI (161) compared with TESE-ICSI with testicular elongated spermatids may further limit the application of ROSI for human fertility restoration. Therefore, further development of these round spermatids or spermatid-like cells into more mature elongated spermatids is crucial for future clinical application.

CONCLUDING REMARKS

As the patient population of pre-pubertal boys who have been offered fertility preservation slowly reaches their reproductive age, the issue of offering fertility restoration becomes more urgent. The research on SSCT, tissue grafting and *in vitro* spermatogenesis has concomitantly matured over the years and currently applications are on the horizon. For SSCT, the proof of principle has been delivered through allotransplantations in monkeys and the safety of the procedure has been extensively studied in mice. Potential for further optimization lays in *in vitro* human SSC propagation to increase their numbers and to purge cell suspensions of cancerous cells prior to transplantation. The use of testicular tissue grafting is also within reach of the clinic, with the existence of an autologous non-human primate model having resulted in healthy offspring. Challenges for grafting still exist with regard to the harvest of sufficient amounts of testicular tissue for grafting, the prevention of apoptosis within these grafts and assessment of the genomic status of the graft derived spermatids, as well as finding ways of preventing reintroduction of cancerous cells to the patient from within the grafted tissues.

Although spermatogenesis *in vitro* or *ex vivo*, either through cell or tissue culture, shows great promise with regard to the controlled production of spermatids, complete spermatogenesis with human tissue or isolated SSCs has not yet been indisputably achieved. Along with major concerns surrounding the genomic stability of haploid spermatid (like) cells generated by *in vitro* spermatogenesis, this technique does not yet seem ready for clinical application.

Despite these challenges, as continuous achievements are made within this field of research, all three avenues towards fertility restoration remain worth pursuing. To ensure progress, it is necessary to explore all options for tissue resources in preclinical research, although we should remain vigilant of the effect the tissue of choice has on study outcomes. In addition, more fundamental research towards identification of reliable *in vivo* and *in vitro* markers for identification and confirmation of testicular cell populations is vital for progress in this field.

For current and future patients, we should seek to offer comprehensible up-to-date and honest information on the progress of potential fertility preservation and restoration strategies and to inquire as to their wishes regarding these procedures.

AUTHOR CONTRIBUTIONS

IS and JM took the lead in drafting this manuscript, all other co-authors (JE, QL, AM, AAM, GH, AP, and CM) wrote sections of this manuscript. AAM, AM, AP, and JE contributed substantially to the cryopreservation section, while QL and GH contributed substantially in the (safety of) *in vitro* or *ex vivo* spermatogenesis. JM designed the figure and table. All authors took part in critical review and revising of the manuscript and approved the final version.

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Microfluidic and Static Organotypic Culture Systems to Support *Ex Vivo* Spermatogenesis From Prepubertal Porcine Testicular Tissue: A Comparative Study

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Background: *In vitro* maturation of immature testicular tissue (ITT) cryopreserved for fertility preservation is a promising fertility restoration strategy. Organotypic tissue culture proved successful in mice, leading to live births. In larger mammals, including humans, efficiently reproducing spermatogenesis *ex vivo* remains challenging. With advances in biomaterials technology, culture systems are becoming more complex to better mimic *in vivo* conditions. Along with improving culture media components, optimizing physical culture conditions (e.g., tissue perfusion, oxygen diffusion) also needs to be considered. Recent studies in mice showed that by using silicone-based hybrid culture systems, the efficiency of spermatogenesis can be improved. Such systems have not been reported for ITT of large mammals.

Methods: Four different organotypic tissue culture systems were compared: static i.e., polytetrafluoroethylene membrane inserts (OT), agarose gel (AG) and agarose gel with polydimethylsiloxane chamber (AGPC), and dynamic i.e., microfluidic (MF). OT served as control. Porcine ITT fragments were cultured over a 30-day period using a single culture medium. Analyses were performed at days (d) 0, 5, 10, 20 and 30. Seminiferous tubule (ST) integrity, diameters, and tissue core integrity were evaluated on histology. Immunohistochemistry was used to identify germ cells (PGP9.5, VASA, SYCP3, CREM), somatic cells (SOX9, INSL3) and proliferating cells (Ki67), and to assess oxidative stress (MDA) and apoptosis (C-Caspase3). Testosterone was measured in supernatants using ELISA.

Results: ITT fragments survived and grew in all systems. ST diameters, and Sertoli cell (SOX9) numbers increased, meiotic (SYCP3) and post-meiotic (CREM) germ cells were generated, and testosterone was secreted. When compared to control (OT), significantly larger STs (d10 through d30), better tissue core integrity (d5 through d20), higher numbers of undifferentiated spermatogonia (d30), meiotic and post-meiotic germ cells (SYCP3: d20 and 30, CREM: d20) were observed in the AGPC system. Apoptosis, lipid peroxidation

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(MDA), ST integrity, proliferating germ cell (Ki67/VASA) numbers, Leydig cell (INSL3) numbers and testosterone levels were not significantly different between systems.

Conclusions: Using a modified culture system (AGPC), germ cell survival and the efficiency of porcine germ cell differentiation were moderately improved *ex vivo*. We assume that further optimization can be obtained with concomitant modifications in culture media components.

Keywords: spermatogonial stem cells, immature testicular tissue, organotypic culture, microfluidic culture, *in vitro* spermatogenesis, fertility preservation, cancer, boys

1 INTRODUCTION

Cryopreservation of immature testicular tissue (ITT) containing spermatogonial stem cells (SSCs) is an ethically accepted and recommended approach (as per several oncology, pediatric and fertility societies recommendations) for fertility preservation (Practice Committee of the American Society for Reproductive Medicine. Electronic address, 2019; Mulder et al., 2021). It can be offered to prepubertal boys at risk of becoming infertile (e.g., following a gonadotoxic treatment for cancerous or benign diseases) with the aim of restoring their fertility if diagnosed with azoospermia when facing a child wish (Wyns et al., 2020).

Fertility preservation programs have been developed since the early 2000s, and to date, the most recent survey showed that more than a thousand boys have participated in Europe, Canada, and the United States (Goossens et al., 2020).

The largest series reporting follow-up data on the reproductive potential of these boys showed that 46% who had undergone a testicular biopsy for fertility preservation before the onset of spermatogenesis suffered from a severely impaired spermatogenesis (azoospermia in 29% of cases) after a median of 6.5 (2.6–14) years from gonadotoxic treatment completion. This makes the development of clinically-applicable fertility restoration methods using cryopreserved ITT an urgent matter in order to give these boys the hope to father their own biological child (Kanbar et al., 2021).

So far, three experimental methods have been considered for fertility restoration with cryopreserved ITT: tissue auto-grafting, SSCs transplantation, or *in vitro* maturation (IVM) of the tissue or the SSCs (Wyns et al., 2020). Autografting of frozen-thawed ITT has already led to the birth of a healthy female baby monkey using graft-derived sperm (Fayomi et al., 2019), while transplantation of SSCs led to offspring in some animal species and ICSI-mediated embryos in monkeys (Hermann et al., 2012). While both transplantation of ITT or SSCs back to the patient present the risk of cancer cell contamination of the tissue or cell suspension, and thus of disease relapse, IVM offers the advantage of circumventing this risk by using *in vitro* generated sperm to obtain embryos *ex vivo*.

Efforts to reproduce spermatogenesis *in vitro* have been ongoing since more than a 100 years (Wyns and Kanbar, 2022) and have been fueled in the past decade by advancements in *in vitro* culture techniques. Among the noteworthy achievements and developments were the creation of *in vitro* culture physical support systems (e.g., hanging drops, metallic wire grids, agarose blocks, meshed membranes, synthetic scaffolds), the development of enriched and complex culture media for metabolic support (e.g., using lymph/plasma clots, serum, growth factors and hormones, and more recently serum-free components) and attempts to the definition of most adequate culture conditions (e.g., sterility, temperature, humidity, gas content, pH, etc.) (Stukenborg et al., 2009; Richer et al., 2020; Wyns and Kanbar, 2022).

Yet, despite long-lasting efforts, IVM of prepubertal testicular tissue from rats (Reda et al., 2016; Matsumura et al., 2021; Saulnier et al., 2021) and larger mammals (Heckmann et al., 2020; Sharma et al., 2022) including humans (de Michele et al., 2017; Medrano et al., 2018; Portela et al., 2019a; Kurek et al., 2021) remained disappointing compared to achievements in mice where fertilization-competent spermatozoa leading to live births were produced (Sato et al., 2011; Yokonishi et al., 2014; Komeya et al., 2016) although the success of *in vitro* spermatogenesis in the latter species seemed to be strain-dependent (Portela et al., 2019b). In the best-case scenario for humans, few haploid germ cells were produced *in vitro* starting from SSCs found within a testicular cell suspension (Abofoul-Azab et al., 2018) or from intact ITT fragments of prepubertal boys (de Michele et al., 2018). Moreover, spermatogonia numbers decreased over the culture period.

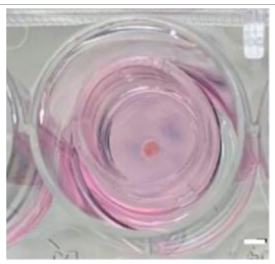
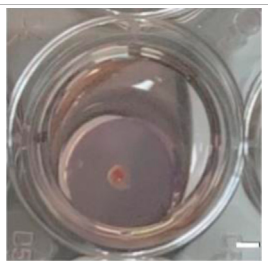
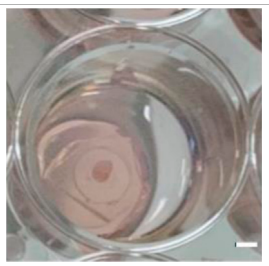
Overcoming these limitations is therefore essential to allow for a potential clinical application of the technique in the future.

Since the early-2000s, tissue engineering technologies have been used to increasingly mimic the *in vivo* conditions notably in terms of tissue/cell perfusion and oxygenation amelioration (Lovett et al., 2009; Place et al., 2017) leading to an improved culture outcome (e.g., viability and function) in thick tissues like liver (Khong et al., 2007) and cardiac muscles (Vollert et al., 2014).

Recently, some of these technologies (bioreactors, microfluidic devices) have also been applied to the culture of testicular tissue fragments (Komeya et al., 2016; Yamanaka et al., 2018; Komeya et al., 2019; Matsumura et al., 2021), isolated seminiferous tubules (Perrard et al., 2016) and testicular cell suspensions (Dores et al., 2015; Baert et al., 2020) by different groups (reviewed by Oliver and Stukenborg, 2020).

The most exciting results in terms of *in vitro* spermatogenesis were recently reported by the group of Ogawa in Japan who implemented microfluidic (MF) systems. Using ITT fragments (0.5–6.5 mm^2) from prepubertal mice, the group demonstrated the successful maturation and long-term (6 months) maintenance of the tissue *in vitro* using a polydimethylsiloxane (PDMS) based MF organotypic culture system (PDMS being a widely used and biocompatible

TABLE 1 | Summary of culture systems used in the experiments.

Static			Dynamic
Porous PTFE Membrane (0.4 µm Pores)	Agarose gel pillars		Microfluidic PDMS based devices
	Without a PDMS component	With a PDMS component	
			
Culture insert PTFE (OT)	Agarose (AG)	Agarose + PDMS cover chip (AGPC)	Microfluidic (MF)

PDMS, Polydimethylsiloxane; PTFE, Polytetrafluoroethylene, Scale bars = 2 mm.

silicone-based and gas-permeable organic polymer). Key findings were the maintenance of the endocrine function (until day 180) and the generation of fertilization-competent haploid germ cells with the birth of healthy progeny (Komeya et al., 2016). Most importantly, when compared to a static agarose gel (AG) organotypic culture system (Sato et al., 2011), the MF system allowed for both an improved tissue survival and a significantly higher number of spermatids and spermatozoa being generated *ex vivo* (Komeya et al., 2016).

Subsequently, the same group later demonstrated that by using a "PDMS cover chip" (PC) to cover and flatten (thus also improving tissue perfusion and oxygenation) the ITT fragment on the classical AG static system, mice spermatogenesis efficiency *in vitro* was also significantly improved (Komeya et al., 2019). However, no comparison with the MF system was done.

The impact of such PDMS-based systems on the IVM of ITT from larger animal species (including humans) remains unknown to date. As marked differences exist between rodent and human spermatogenesis in terms of regulation (e.g., CXCL12-CXCR4 expression, CSF1R localization, Hedgehog/NOTCH signaling) (Guo et al., 2018) and efficiency (Fayomi and Orwig, 2018) it is important to determine whether results obtained in mice can be translated to other species.

In this study we therefore aimed to compare four different organotypic tissue culture systems (3 static and 1 dynamic) using fresh ITT fragments from the domestic pig (a species that has a very close reproductive tract anatomy and physiology to that of humans, with a poorly efficient spermatogenesis) (Swindle and Smith, 1998; Almeida et al., 2006; Groenen et al., 2012), in order to help defining to which extent the type of culture system influences the maturation of large mammalian ITT *in vitro*.

2 MATERIALS AND METHODS

2.1 Tissue Culture Systems

Four culture systems were compared in this study as depicted in **Table 1**.

2.1.1 Culture Inserts (OT)

The culture inserts (PICM01250, Merck) used were identical to those previously used by our group to successfully generate haploid human germ cells *in vitro* (de Michele et al., 2018). This system (OT) served therefore as control for all other culture systems. Tissue inserted in this system are in immediate contact with the culture medium (deposited on the thin PTFE membrane) (**Figure 1A**).

2.1.2 Agarose Gel Pillars (AG)

To make the agarose gel pillars (1.5% w/v), agarose powder (A6013, Sigma Aldrich) was dissolved in distilled water and autoclaved for 30 min. A 5 mm thick layer of agarose gel was formed after pouring and cooling of 30 ml of the agarose liquid solution into a 9.4 cm glass Petri dish. The gel was then perforated using a 10 mm diameter punch biopsy (69036-100, Harris Uni-core) into multiple cylinder-shaped pillars. Pillars were then placed in the wells of 24-well culture plates (CLS3527, Corning Star). They were entirely covered with culture medium and left in the incubator overnight. The next day, at the start of the experiments, the old medium was replaced by fresh medium. Each agarose pillar was then loaded with one testicular tissue fragment. Tissue inserted in this system were not in direct contact with the culture medium but with the agarose surface (**Figure 1B**).

2.1.3 Agarose Gel Pillars With PDMS Cover Chip (AGPC)

For the AGPC culture system, the PC chip (with a ≃ 170 µm deep chamber) was added on top of the tissue fragments, thus evenly spreading the tissue deposited on the same agarose pillars described above (**Figure 1C**).

2.1.4 Microfluidic System (MF)

For the MF culture system, tissue fragments were deposited inside the tissue chamber (**Figure 1D**) of the plasma pre-treated MF chips (SB plasma treater, BlackHoleLab, France). The patterned PDMS chips were then bonded to a microscope glass (VWR, Belgium) and connected to the medium inlet tube (374080, Thermofisher) and

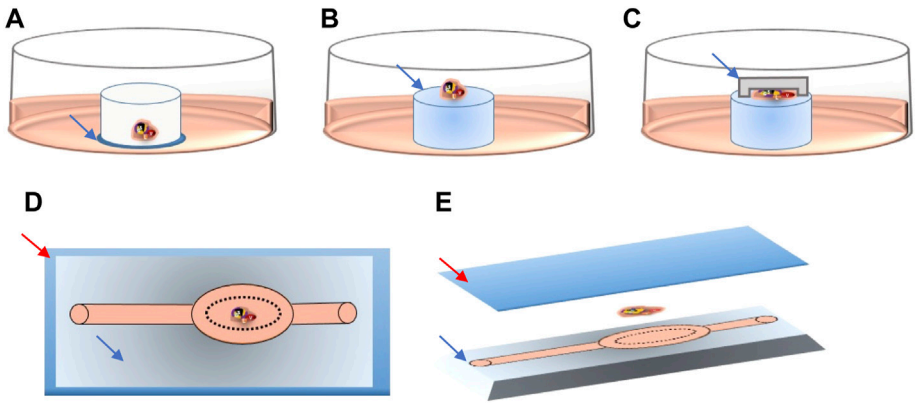


FIGURE 1 | Schematic detail of tissue location in the different culture systems. Culture medium is shown in pink in all systems. **(A)** Blue arrow pointing to the thin PTFE membrane, directly contacting the culture medium. **(B)** Blue arrow pointing to the agarose gel pillar. **(C)** Blue arrow pointing to PDMS cover chip deposited on top of the tissue fragment. **(D)** Upper view of the PDMS chip, red arrow pointing to the glass slide, blue arrow to the patterned PDMS chip. **(E)** 3D lateral view of the PDMS chip showing the glass slide (red arrow), tissue fragment and patterned PDMS chip (blue arrow).

syringe-pump outlet (Aladdin NE-1200-EM, WPI, UK) using 1.0/2.0 mm PVC tubes (10192251, FischerSci) (**Figures 1D,E**).

2.2 Design and Fabrication of the PDMS Components for the AGPC and MF Systems

The MF devices and PC chips were fabricated using the photolithography and soft lithography techniques. Protocols used for fabrication were modified from those described by the group of Ogawa (Komeya et al., 2016; Komeya et al., 2019) to achieve a small device that could fit on a standard microscope slide (75 × 25 mm), with only one inlet, and an elliptic-shaped chamber that is perfused from all sides (**Supplementary Figure 1**).

Briefly, the PDMS prepolymer and curing reagent (Sylgard 184, Dow) were mixed at a 10:1 weight ratio. Then the mixture (10 g for PC and 30 g for MF) was degassed and poured over the specific mold masters (**Supplementary Figure 1B, F**), which were then placed in an oven at 80 °C for 45 min for curing. After cooling, the solidified PDMS was peeled off from the master mold and was cut using a surgical blade into individual chips. PC and MF chips were about 1 and 3 mm thick, respectively.

Mold masters used for soft lithography were produced as previously reported using conventional photolithography techniques (McDonald et al., 2000). Briefly, the material of the master mold, a negative photoresist (SU-8, 2100; MicroChem Co.) was poured on a 4-inch wafer and spincoated over it to evenly achieve the target thickness of 170 µm over the wafer. After prebaking, the coated wafer was exposed to ultraviolet light (at 365 nm) that was administered through the different photomasks, and then postbaked at 100°C. The baked mold masters were immersed in an SU8-Developer (Y020100, MicroChem) in an ultrasonic bath environment for 20 min and were then rinsed in isopropanol. Wafers were finally silanized prior to use.

The photomasks were designed with CAD software (AutoCAD 2019, academic free license; Autodesk Inc., San Rafael, California) and fabricated with a laser lithography system (JD photomask, United Kingdom).

2.3 Animal Tissue and Culture Method

Piglet testes (approx. 5–9 days old) were recovered as a byproduct of castration from a local Belgian swine farm. Briefly, testes (n = 3) were decapsulated, and cut into small fragments (0.5–1 mm^3) and cultured in the different culture systems at 34°C in 5% CO_2. Static culture systems were all placed in 24-well plates.

The amount of medium added was 300 µl for the OT, AG, and AGPC groups with change of medium every 4–5 days. For the MF group, medium was drawn through the chip outlet using the syringe pump at a speed of 0.05 µl/min. Fresh culture medium was added to the inlet reservoir in the MF group every 4–5 days. All PDMS and PVC components used for the culture experiments were initially sterilized in an autoclave for 20 min.

The culture medium used in all experiments was Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with Knockout serum replacement (KSR) 10%, FSH (5 IU/L), and antibiotics (Gentamicin 10 µg/ml and Ceftazidime 3 mg/L) as previously described by our group (de Michele et al., 2018).

2.4 Live Imaging of the Tissue in Culture

Tissues in culture were observed every 5 days with an inverted microscope (Oxion Inverso, EUROMEX, Netherlands) and serial pictures were taken to evaluate the growth of tissue fragments. Images were then processed using the ImageJ software (Schneider et al., 2012) to determine the tissue surface and its 2D evolution through the culture period.

2.5 Retrieval of Cultured Tissue Fragments and Supernatants

Cultured ITT fragments from three piglets were harvested for analyses on days 5, 10, 20, and 30 and then fixed in paraformaldehyde 4% (VWR, Belgium) overnight before being

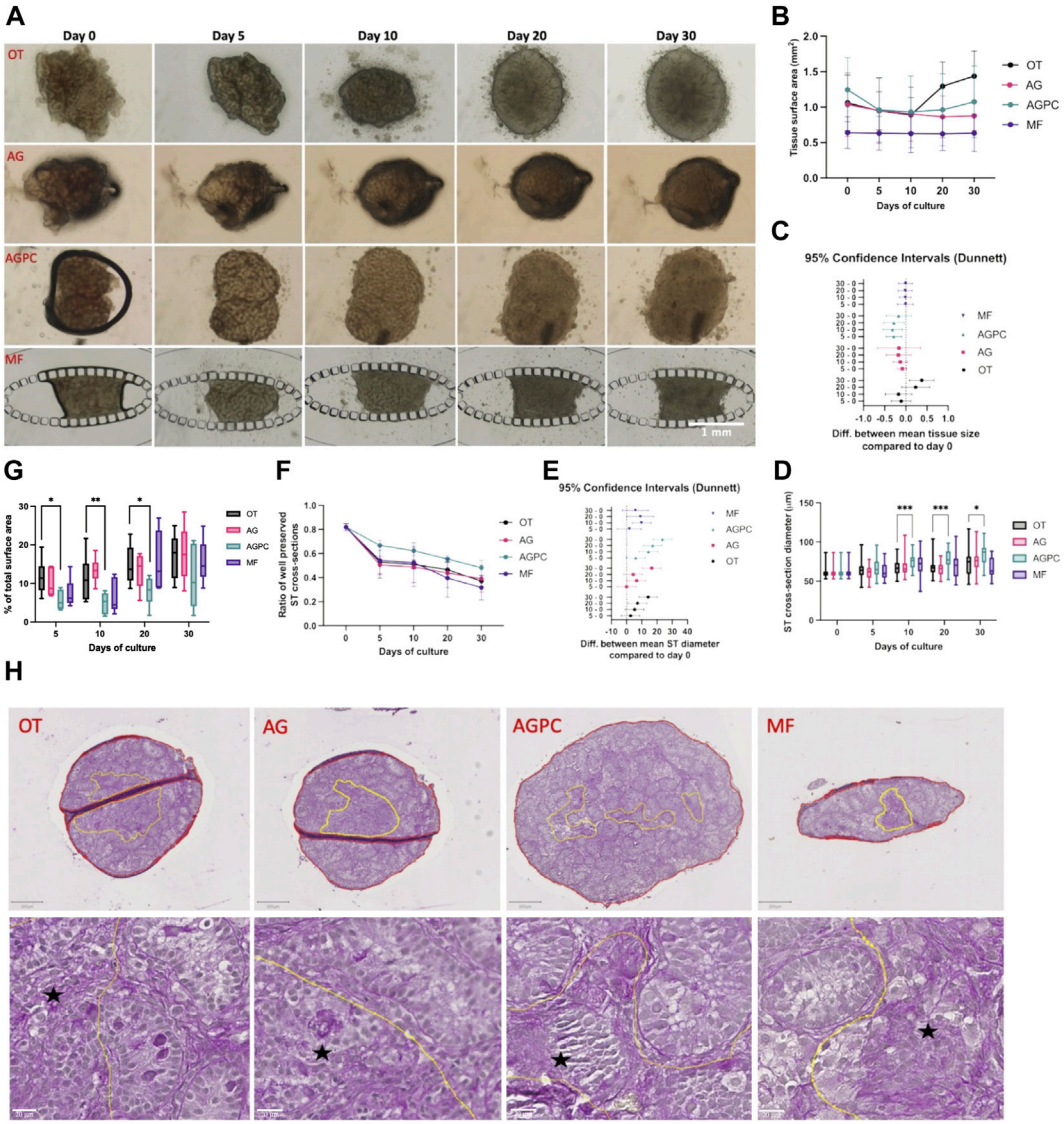


FIGURE 2 | Evolution of tissue size change, ST cross-section diameter and integrity, and tissue core degeneration over the culture period. **(A)** Photographic images of tissue fragments in the different systems from day 0 to day 30. **(B)** Evolution of mean tissue size over time in all systems. **(C)** Difference in mean tissue size between day 30, 20, 10 and day 0. **(D)** Evolution of mean ST cross-sections diameter in the different systems over the culture period. **(E)** Difference in mean ST cross-section diameter between day 30, 20, 10 and day 0. **(F)** Evolution of mean ST cross-sections integrity in the different systems over the culture period. **(G)** Change in the percentage of the central degenerative area (among total tissue area) in the different systems over the culture period. **(H)** Assessement of the integrity of the tissue core on PAS stained slides at day 30. Yellow lines: zone(s) with poor core integrity. Red line(s): total tissue surface area. Black star: areas with altered tissue architecture. OT, culture insert PTFE; AG, Agarose; AGPC, Agarose + PDMS cover, MF, Microfluidic.

embedded in paraffin. Five-micrometer-thick sections on Superfrost® Plus slides (VWR, Belgium) were used for histology, immunohistochemistry (IHC) and immunofluorescence (IF). Supernatants were retrieved every four to 5 days and stored at −80°C.

2.6 Histological and Immunohistochemical Analyses

For histological examination, two sections showing the largest cut surface were made for each specimen and stained with Periodic acid-Schiff (PAS) to assess seminiferous tubule (ST) cross-sections (aspect ratio of 1–1.5) for their integrity based on a previously described score (de Michele et al., 2017; de Michele et al., 2018) (**Supplementary Figure 2A**) and to assess tissue core integrity by measuring centrally degenerated and total tissue areas. Results were reported as the ratio of well-preserved over total ST cross-sections (ST integrity) and as the percentage of centrally degenerated among total tissue area (Tissue core integrity). The evolution of well-preserved ST cross-sections mean diameter was also studied (on 40 ST cross-sections per timing and per system) by measuring their largest

diameters (inner to inner measurement from the basal lamina).

Immunohistochemistry (IHC) was performed for the identification of undifferentiated spermatogonia (anti-PGP9.5), Sertoli cells (anti-SOX9), Leydig cells (anti-INSL3), meiotic germ cells (anti-SYCP3), post-meiotic germ cells (anti-CREM) and for the assessment of apoptosis (anti-cleaved-caspase-3) and oxidative stress (Lipid peroxidation biomarker anti-MDA) according to protocols previously described (de Michele et al., 2018; Del Vento et al., 2019; Vermeulen et al., 2019).

Duplex immunofluorescence (IF) imaging was performed to identify proliferating germ cells (anti-Ki67, anti-VASA) and to confirm the identification and location of meiotic (anti-VASA+ and anti-SYCP3+) and post-meiotic (anti-VASA+, anti-CREM+) germ cells. All analyses were performed on two randomly selected sections at least 50 µm apart.

For histology and IHC images, slides were scanned at 400x using a Leica SCN400 slide scanner (Leica Microsystems, Wetzlar, Germany) and for IF using a Zeiss Axio Scan. Z1 (Carl Zeiss, Göttingen, Germany). Quantification was done on entire tissue sections using Qupath software (Bankhead et al., 2017) for all the studied markers. Results for intratubular markers (PGP9.5, SOX9, VASA+/Ki67+, SYCP3 and CREM) were reported as the number of positive cells per ST cross-section (de Michele et al., 2018). Results for Cleaved caspase-3 and INSL3 were reported as the percentage of positive cells among the total cell and total interstitial cell populations, respectively. Results for MDA were reported as a HistoScore (Jensen et al., 2017). The HistoScore was calculated by a semi-quantitative assessment including both the intensity of staining (stained membranes graded as 0, non-staining; 1, weak; 2, median; or 3, strong) and the percentage of positive cells (**Supplementary Figure 2B**). The following formula was used; HistoScore = ((1 x % weakly stained cells) + (2 x % moderately stained cells) + (3 x % strongly stained cells). The total surface area of tissue sections was considered for analyzes of tissue core integrity, cleaved caspase-3, INSL3 and MDA.

For positive IHC tissue controls, we used neonatal porcine ITT (PGP9.5), tonsil (cleaved caspase-3), mature porcine testicular tissue (for VASA, Ki67, INSL3, SYCP3, and CREM), and human placenta (MDA). For negative controls the primary antibodies were omitted (**Supplementary Figure 4**).

All primary and secondary antibodies used in these experiments are detailed in **Supplementary Table 1**.

2.7 ELISA Analyses

Reagents of testosterone ELISA kits (ABNOKA2349, Abnova, Taiwan) were prepared following the manufacturer's instructions. Supernatants were homogenized and centrifuged before analyses. A 50-fold dilution was necessary for supernatants of the OT, AG, and AGPC, groups starting day 10, while a five-fold dilution was applied to supernatants of day 5 samples from all groups and to the MF supernatants at all timepoints. Absorbance was read at 450 nm by the iMark™ microplate absorbance reader (1681135, Bio-Rad).

2.8 Statistical Analyses

Statistical analyses were performed with the GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, United States). Data are presented as mean ± SD. A mixed-effects analysis followed by a Dunnett's test for multiple comparison (n = 3 for histology, immunohistochemistry, and ELISA measures) were performed on normally distributed data (with or without a log-transformation) to analyze the effect of culture time and culture system on the different dependent variables. Random effects (within subjects' variability) for the different piglets were also analyzed within the model and only reported when significant. OT system and day 0 results were used as control. Pearson's r was used for correlation analyses. Statistically significant results were reported as * ($p \leq 0.05$), ** ($p \leq 0.01$), ***($p \leq 0.001$) and **** ($p \leq 0.0001$) on graphs.

An additional post-hoc multiple comparison (Tukey's HSD) analysis comparing all systems among each other (and not to control alone) was also performed.

3 RESULTS

3.1 Evolution of Tissue Size, Seminiferous Tubule Diameter and Integrity, and Tissue Core Integrity in Culture.

The evolution of tissue size over the culture period in the different systems is shown in (**Figures 2A,B**). Both culture time ($p = 0.000$) and the type of system ($p = 0.01$) were shown to have a significant impact on the results.

By day 30, fragments in the OT (1.3 ± 0.12 mm^2) system had increased in size (p = 0.01) while those in the AG (0.86 ± 0.21 mm^2), AGPC (1.07 ± 0.5 mm^2) and MF (0.63 ± 0.26 mm^2) did not significantly change when compared to day 0 (OT: 1.06 ± 0.39 mm^2, AG: 1.16 ± 0.24 mm^2, AGPC: 1.24 ± 0.45, MF: 0.64 ± 0.22 mm^2). The mean changes in fragments' area between the different timepoints and the beginning of the culture (day 0) are shown in **Figure 2C**.

The mean diameter of ST cross-sections statistically increased between day 0 (61.2 ± 6.5 µm) and the end of the culture in all systems ($p < 0.000$) (at day 30, OT: 75.3 ± 18.1 µm, AG: 77.7 ± 15.7 µm and AGPC: 84.9 ± 13.6 µm) except for MF (66.9 ± 12.9 µm) where the increase was only significant till day 20 (71 ± 13 µm, p = 0.04). The tissue fragments cultured in the AGPC system had a significantly greater mean ST diameter at days 10 ($p < 0.000$), 20 ($p < 0.000$) and 30 ($p = 0.02$) when compared to control (**Figure 2D**). The mean changes in ST diameters between the different timepoints and the beginning of the culture (day 0) are shown in **Figure 2E**.

The analysis for ST integrity was performed on a total of 931 ST cross-sections (OT: 245, AG: 254, AGPC: 228, MF: 204). Culture time ($p < 0.000$) and type of system ($p = 0.04$) were shown to have a significant impact on the results.

There was a significant decrease over time in the ratio of well-preserved ST-cross sections in all systems. When compared to day 0 (0.81 ± 0.03), at day 30 the ratio of well-preserved STs was: 0.37 ± 0.01 (OT, $p < 0.000$), 0.39 ± 0.11 (AG, $p < 0.000$), 0.48 ±

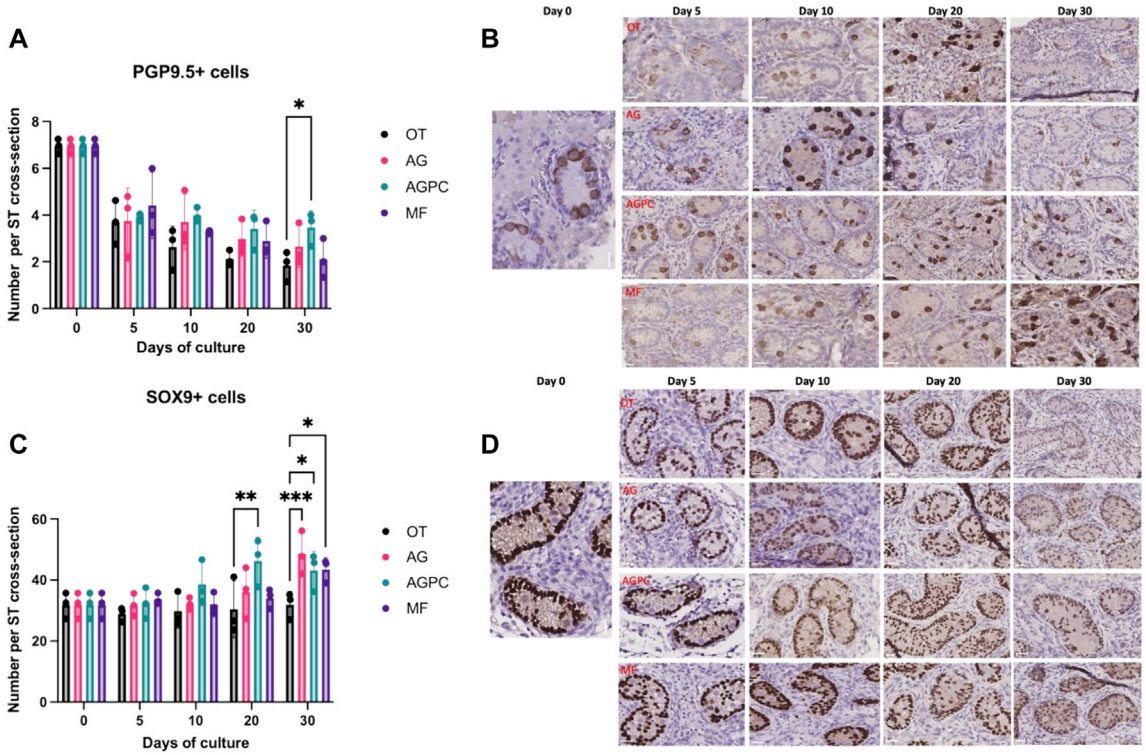


FIGURE 3 | Evolution of undifferentiated spermatogonia and Sertoli cell numbers per ST cross-section over the culture period. **(A)**: Evolution of the number of PGP9.5 positive cells per ST cross-section over the culture period. **(B)**: PGP9.5 staining on immunohistochemistry. **(C)**: Evolution of the number of SOX9 positive cells per ST cross-section over the culture period. **(D)**: SOX9 staining on immunohistochemistry. OT, culture insert PTFE; AG, Agarose; AGPC, Agarose + PDMS cover; MF, Microfluidic. Images shown at ×400 magnification. Scale bars = 20 µm.

0.06 (AGPC, $p = 0.000$) and 0.31 ± 0.1 (MF, $p < 0.000$). There were no significant differences between any of the systems at any timepoint (**Figure 2F**). No correlation was found between the mean evolution of tissue fragments' size and ST integrity in any of the studied systems (OT: r = 0.23, $p = 0.41$, AG: r = 0.48, $p = 0.41$, AGPC: r = 0.56, $p = 0.32$, MF: r = 0.77, $p = 0.12$).

We found a statistically significant effect of time ($p < 0.000$) and type of culture system ($p = 0.001$) on the percentage of tissue core degeneration (**Figures 2G,H**). Central tissue degeneration was evidenced starting day 5 and increased in all systems reaching mean peak values at day 30 (OT: 17.1 ± 5.79%, AG: 17.75 ± 7.02%, AGPC: 11.41 ± 8.23%, MF: 15.65 ± 5.53%). Tissue fragments cultured in the AGPC system had a significantly smaller percentage of tissue area with core degeneration at days 5 ($p = 0.03$), 10 ($p = 0.008$) and 20 ($p = 0.03$) when compared to control (**Figure 2G**). The total tissue surface area analyzed was 47.7 mm^2 (OT: 14.1, AG: 11.9, AGPC: 13.4, MF: 8.2 mm^2).

Based on the multiple comparison using Tukey's test among all four systems, tissue fragments cultured with the AGPC system also showed a larger mean ST diameter, when compared to AG (at day 10, $p = 0.01$ and 20, $p < 0.000$) and when compared to MF (at day 30, $p < 0.000$), and an improved tissue core integrity when compared to AG (at day 10, $p < 0.000$) (**Supplementary Figure 5**).

3.2 Undifferentiated Spermatogonia and Sertoli Cell Numbers

The analysis for PGP9.5 cells was performed on a total of 1408 ST cross-sections (OT: 354, AG: 388, AGPC: 398, MF: 268). We found a statistically significant effect on the average number of undifferentiated spermatogonia (PGP9.5+) per ST cross-section of both the culture time ($p < 0.000$) and the culture system ($p = 0.02$) (**Figures 3A,B**).

Compared to day 0 (6.94 ± 0.31), there was a statistical decrease in the number of PGP9.5 + cells at all time points and in all systems ($p < 0.000$). However, at day 30 (OT: 1.84 ± 0.64, AG: 2.65 ± 0.88, AGPC: 3.72 ± 0.70, MF: 2.11 ± 0.83), the number of PGP9.5 + cells/ST cross-section was found to be higher in the AGPC system in comparison to OT ($p = 0.01$) (**Figure 3A**).

We found a statistical effect on the average number of Sertoli cells (SOX9+) per ST cross-section (analysis was performed on a total of 882 ST cross-sections, OT: 254, AG: 204, AGPC: 234, MF: 190) of both the culture time ($p < 0.000$) and the culture system ($p = 0.002$) (**Figures 3A,B**).

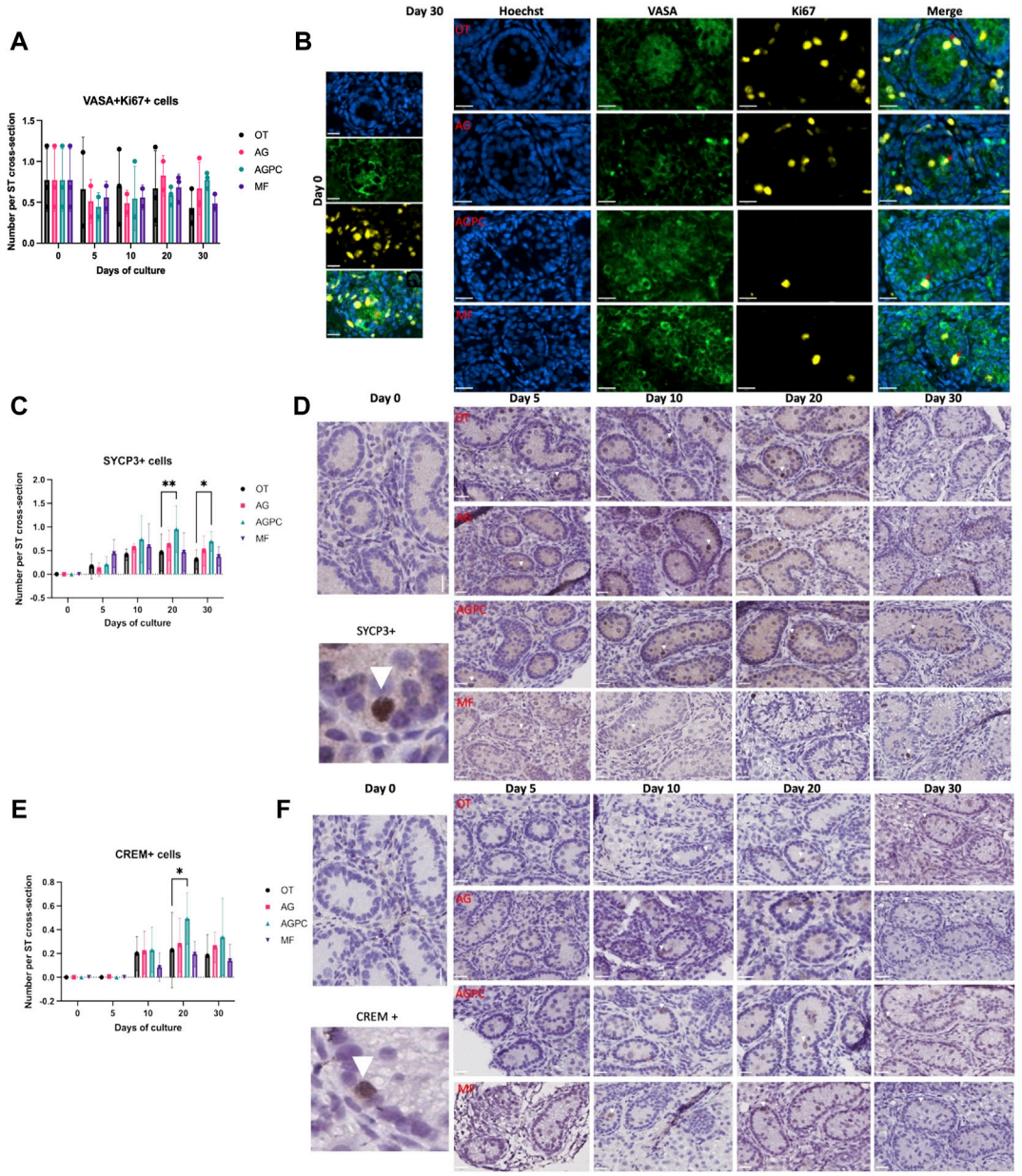


FIGURE 4 | Evolution of germ cell proliferation and differentiation over the culture period. **(A)** Evolution of the number of proliferating germ cells (VASA + Ki67+) cells per ST cross-section over the culture period. **(B)** Immunofluorescence duplex staining for VASA/Ki67, red arrows indicate proliferating germ cells. **(C)** Evolution of the number of meiotic (SYCP3+) cells per ST cross-section over the culture period. **(D)** SYCP3 staining on immunohistochemistry, white arrowheads show SYCP3+ staining. **(E)** Evolution of the number of post-meiotic (CREM+) cells per ST cross-section over the culture period, **(F)** CREM staining on immunohistochemistry, white arrowheads show CREM + staining. OT, culture insert PTFE; AG, Agarose; AGPC, Agarose + PDMS cover; MF, Microfluidic. Images shown at ×400 magnification. Scale bars = 20 µm.

Between days 0 (31.82 ± 4.25) and 30, the number of Sertoli cells (SOX9+) significantly increased in AG (48.64 ± 7.11, p = 0.000), AGPC (43.1 ± 6.14, p = 0.001) and MF (43.44 ± 3.86, p = 0.001) systems but not in OT (31.93 ± 4.35). At day 30, there were significantly more SOX9+ cells/ST cross-section in the AG (p = 0.001), AGPC (p = 0.03) and MF (p = 0.02) systems in comparison to control (OT) (**Figures 3C,D**).

Also, the multiple comparison analysis using Tukey's test showed that the number of SOX9+ cells/ST was significantly higher in the AGPC group in comparison to MF (day 20, p = 0.03) (**Supplementary Figure 5**).

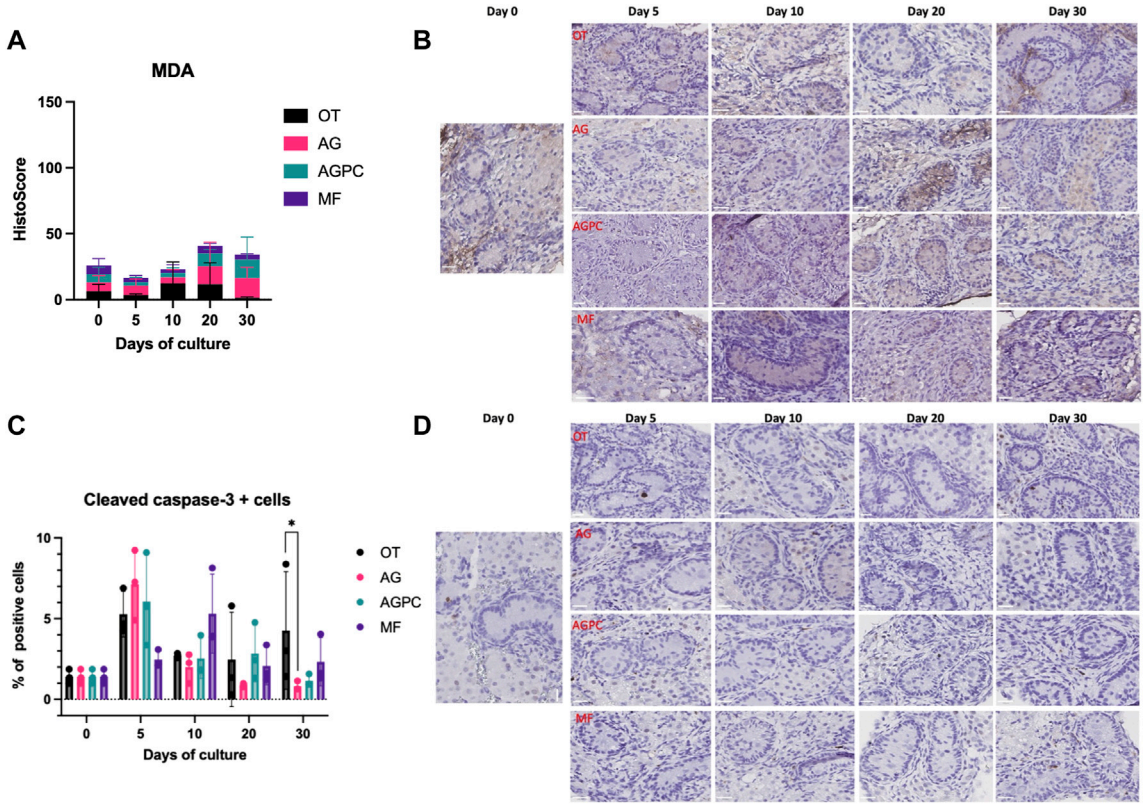


FIGURE 5 | Evolution of apoptosis and oxidative stress markers over the culture period. **(A)** Evolution of the MDA staining (HistoScore) levels over the culture period. **(B)** MDA staining on immunohistochemistry. **(C)** Evolution of the percentage of cleaved caspase-3 positive cells over the culture period. **(D)** Cleaved caspase-3 immunohistochemistry. OT, culture insert PTFE; AG, Agarose; AGPC, Agarose + PDMS cover; MF, Microfluidic. Images shown at ×400 magnification, Scale bars = 20 µm.

3.3 Germ Cell Proliferation and Differentiation

Both the culture time and the culture system did not have a significant impact on germ cell proliferation (VASA + Ki67 + cells). Changes in the mean number of VASA + Ki67 + per ST cross-section were insignificant between day 0 (0.77 ± 0.38) and day 30 (OT: 0.43 ± 0.21, AG: 0.66 ± 0.32, AGPC: 0.77 ± 0.1, MF: 0.48 ± 0.1) (**Figures 4A,B**). The analysis for VASA + Ki67 + cells was performed on a total of 1107 ST cross-sections (OT: 307, AG: 285, AGPC: 335, MF: 180).

Spermatocytes (SYCP3+) were absent at day 0 in ITT of all piglets (**Figures 4C,D**). Culture time ($p < 0.000$) and the type of system ($p = 0.006$) were shown to have a significant impact on their generation.

Spermatocytes were observed as early as day 5, and their numbers increased significantly starting day 10 in all four systems (OT: 0.41 ± 0.26, $p = 0.04$, AG: 0.55 ± 0.1, $p = 0.003$, AGPC: 0.74 ± 0.49, $p < 0.000$, MF: 0.58 ± 0.4, $p = 0.04$) (**Figure 4C**). At days 20 and 30, the number of SYCP3+ cells/ST cross-section in the AGPC system (d20; 0.95 ± 0.49, d30; 0.69 ± 0.21) was statistically higher than in control (d20; 0.46 ± 0.39, d30; 0.31 ± 0.21) (d20; $p = 0.007$, d30; $p = 0.04$) (**Figures 4C,D**). The analysis for SYCP3 was performed on a on a total of 1524 ST cross-sections (OT: 416, AG: 362, AGPC: 457, MF: 289).

Post-meiotic germ cells (CREM+) were not observed on day 0 in any of the piglets (**Figures 4E,F**). They started to develop at day 10 in the different systems (except for a few positive cells observed starting day 5 in the AGPC group). At day 30, the average number of CREM + cells per ST cross-section was: OT (0.18 ± 0.17), AG (0.25 ± 0.13), AGPC (0.34 ± 0.32) and MF (0.14 ± 0.13).

Although there was a higher number of CREM + cells/ST cross-section in the AGPC group at day 20 in comparison to control ($p = 0.03$), there were no differences observed between the four systems at day 30. Only culture time ($p < 0.000$) and not the type of culture system (=0.06) had an impact on their overall development.

The analysis for CREM was performed on a total of 1293 ST cross-sections (OT: 405, AG: 295, AGPC: 318, MF: 275).

The double IF staining preformed for SYCP3 and CREM with VASA confirmed the expression of both protein markers in differentiating germ cells (**Supplementary Figure 3**).

3.4 Oxidative Stress and Apoptotic Cell Death

Based on the HistoScore evaluation, we found that levels of MDA (**Figures 5A,B**) did not vary significantly neither over the culture

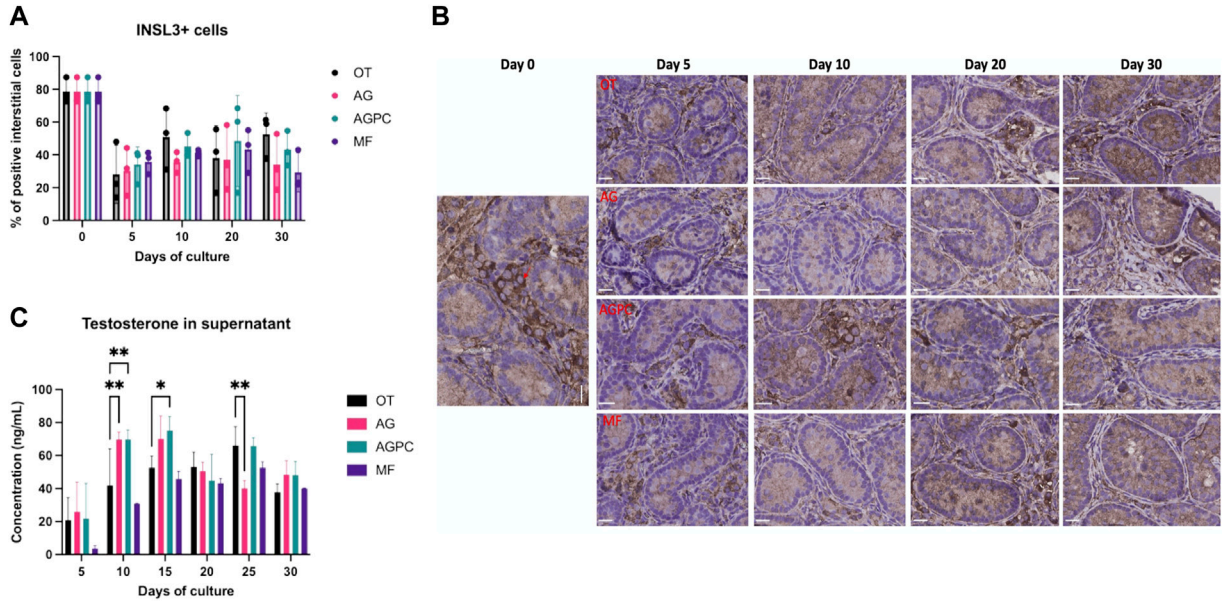


FIGURE 6 | Evolution of Leydig cell numbers and function over the culture period. **(A)** Evolution of the percentage of INSL3+ interstitial cells over the culture period. **(B)** INSL3 staining on immunohistochemistry. Red arrow shows a positive cell. **(C)** Evolution of testosterone secretion over the culture period. OT, culture insert PTFE; AG, Agarose; AGPC, Agarose + PDMS cover; MF, Microfluidic. Images shown at ×400 magnification, Scale bars = 20 µm.

period nor among the different systems. There was no difference between the HistoScore value for MDA level at day 0 (6.45 ± 5.28) and at day 30 for the different systems (OT: 1.41 ± 0.72, AG: 14.9 ± 8.19, AGPC: 13.95 ± 17.21, MF: 3.96 ± 0.63). The total tissue surface area analyzed was 39.95 mm^2 for MDA assessment (OT: 10.84, AG: 9.47, AGPC: 10.82, MF: 8.82 mm^2).

The percentage of cleaved caspase-3 positive cells (**Figures 5C,D**) did not vary with the type of culture system but was significantly influenced by the culture time ($p < 0.000$). Compared to day 0 (1.4 ± 0.44%), a significant peak in apoptosis was noted at day 5 in the static systems (OT: 5.28 ± 1.41%, $p = 0.02$; AG: 7.13 ± 2.16%, $p < 0.000$, and AGPC: 6.07 ± 6.87%, $p = 0.005$) and at day 10 in the dynamic system (MF: 5.31 ± 2.45%, $p = 0.01$) (**Figure 5D**). At day 30, the percentage of cleaved caspase-3 positive cells was statistically lower in the AG system in comparison to control ($p = 0.006$). The total tissue surface area analyzed was 41.1 mm^2 for cleaved caspase-3 evaluation (OT: 9.73, AG: 11.23, AGPC: 12.41, MF: 7.74 mm^2).

3.5 Leydig Cell Numbers and Testosterone Secretion

We found a statistically significant effect of time ($p < 0.000$) on the number of Leydig cells (INSL3+) though the effect of the type of culture system was not significant (**Figures 6A,B**).

At day 0, INSL3+ cells represented an average of 78.66 ± 7.67% of all interstitial cells. By culture day 5, the percentage of INSL3+ stained cells in the interstitium significantly dropped in all systems (OT: 28.11 ± 17.7, AG: 30.38 ± 14.6, AGPC: 34.2 ± 10.6, MF: 35.9 ± 6.83%) and remained unchanged till day 30 (**Figure 6A**). The total tissue surface area was 45.11 mm^2 for INSL3 analyses (OT: 13.9, AG: 10.51, AGPC: 12.8, MF: 7.9 mm^2).

We found a significant effect of both culture time ($p < 0.000$) and the culture system ($p = 0.01$) on the testosterone concentrations in culture supernatants. Testosterone secretion increased in all systems (**Figure 6C**). When compared to day 5, testosterone levels at the end of the culture period (day 30) were significantly higher in AG: 48.4.62 ± 8.5, $p = 0.01$, AGPC: 48.2 ± 8.4, $p = 0.006$, and MF: 40.1 ± 0.3 ng/ml, $p < 0.000$ but not in OT: 37.9 ± 5 ng/ml. At day 30, there were no significant differences in testosterone concentrations between any of the culture systems (**Figure 6C**). Peaks for testosterone levels were observed at days 15 (for AG: 70.1 ± 13.8 and AGPC: 75.2 ± 8.4 ng/ml) and 25 (for OT: 66 ± 11.4 and MF: 52.7 ± 3.5 ng/ml).

When all four systems were compared among each other (Tukey's test), testosterone levels were found statistically higher in the AGPC group compared to MF at days 10 ($p < 0.000$) and 15 ($p = 0.01$) and compared to AG on day 25 ($p = 0.007$) (**Supplementary Figure 5**).

No correlation was found between the % of INSL3+ cells and testosterone in any of the studied systems (OT: r = 0.03, $p = 0.93$, AG: r = -0.13, $p = 0.67$, AGPC: r = 0.16, $p = 0.62$, MF: r = 0.06, $p = 0.87$).

4 DISCUSSION AND CONCLUSION

In 2018 we demonstrated for the first time that haploid human germ cells (round spermatids) can be generated *in vitro* (after 16 days) when ITT fragments were cultured on PTFE culture

inserts (OT) using an enriched culture medium containing KSR 10% and FSH 5 IU/L (de Michele et al., 2018). This was an important first step for the future clinical translation of IVM as a fertility restoration strategy.

As these haploid cells were generated in low numbers, and as murine spermatogenesis *ex vivo* was enhanced when the type of physical culture approach was modified (Komeya et al., 2016; Komeya et al., 2019), it became clear that further studies exploring the impact of the type of culture system, regardless of the culture media components, on the spermatogenic process *ex vivo* were needed.

Due to the scarcity of human ITT available for research, in this work, we chose the porcine model, a large mammal closely related to humans (Swindle and Smith, 1998; Meurens et al., 2012) to study the impact of four different culture systems (OT, AG, AGPC and MF) on the outcome of IVM of ITT in terms of tissue growth, survival and function, including germ cell differentiation. This makes this study the first to report the application of PDMS-based microfluidic (MF) and static (AGPC) organotypic tissue culture systems to ITT of a species that is different from rodents (i.e., mice, rats).

The serial imaging performed in our study was very informative in following the evolution of ITT fragments over time. However, we did not observe a positive relationship between the tissue fragment's growth and overall ST integrity. Indeed, the increase in tissue surface area noted for the OT group did not correlate with an improved outcome in terms of ST integrity. Such enlargement could be due either to cytotoxic cell edema (Liang et al., 2007), fibrosis-related extracellular matrix remodeling (Bigaeva et al., 2019) or to the fact that cells attach and spread better to the PTFE membrane in the OT system in contrast to agarose and PDMS that are both characterized by low to no cell adhesion (Chuah et al., 2015; Cambria et al., 2020).

The overall presence of tissue core degeneration, loss of both the ST integrity and undifferentiated spermatogonia numbers, as well as the increase in apoptosis over the culture period were disappointing, although not surprising. Such phenomena are still a hurdle in *in vitro* experiments across all species, including mice, rats, non-human primates, and humans (Reda et al., 2016; de Michele et al., 2017; Medrano et al., 2018; Portela et al., 2019a; Portela et al., 2019b; Abe et al., 2020; Heckmann et al., 2020; Kurek et al., 2021; Sharma et al., 2022).

A damaged testicular niche environment (demonstrated by the loss of basal membrane protein LAMA1 *in vitro*) (Kurek et al., 2021) and the massive inflammatory reaction described during organotypic culture (Abe et al., 2020) could both participate to the worsening of the tissue evolution in culture. These factors will need to be considered in future organotypic culture experiments.

However, albeit the decrease in the ratio of well-preserved STs and in undifferentiated spermatogonia numbers, some STs were able to grow (based on the observed increase in diameters over the culture period) and to support undifferentiated spermatogonia proliferation and differentiation. Similarly, Sertoli cell (SOX9+) numbers per ST cross-section also rose and testosterone secretion increased reaching levels up to 100 times higher than normal physiological serum levels (Colenbrander et al., 1978; Bonneau et al., 1987). All these changes are the hallmark of the pubertal transition *in vivo* in pigs (Franca et al., 2000; Koskenniemi et al., 2017) and confirm the successful maturation of the neonatal ITT *in vitro*.

Similar to other studies (de Michele et al., 2018; Heckmann et al., 2020; Sharma et al., 2022), germ cell differentiation progressed as expected (first SYCP3 and then CREM) and was accelerated *in vitro*. While both spermatocytes and presumptive spermatids were observed in all systems, their numbers per ST cross-section were higher in the AGPC group. Also, CREM + cells started appearing at day 5 in the AGPC system, earlier than observed in OT, AG, and MF. Both the accelerated development of presumptive spermatids and their presence in higher numbers in the AGPC system could be attributed to an improvement in nutrient diffusion linked to the flattening of the tissue (and thus the delivery of crucial factors/hormones to a broader surface of tissue) as evidenced by the smaller percentage of degenerative tissue core area observed. Such observations are similar to what was previously reported by the group of Ogawa (Kojima et al., 2018; Komeya et al., 2019).

The early reduction and subsequent stabilization in INSL3+ LC numbers together with the increase in testosterone secretion over the culture period were an interesting finding.

INSL3 is a peptide that is secreted by both fetal and adult-type LCs and is an accurate reflector of LCs' differentiation status and of their absolute numbers (Ivell et al., 2013). As fetal LCs are known to peak in numbers shortly after birth and then involute as they are progressively replaced by testosterone-secreting adult-type LCs (immature and then mature LCs as puberty progresses) (Van Straaten and Wensing, 1978; Griswold and Behringer, 2009), we assume that a similar phenomenon might have occurred in our experiments *in vitro*. However, as the INSL3+ cells did not re-increase in numbers (as observed during puberty), it is possible that adult LC proliferation was limited in our experiments. Because LCs play a pivotal role in spermatogenesis, their function, and differentiation status in *ex vivo* ITT culture experiments should be better explored in future studies.

Concerning the lipid peroxidation markers, the reactive aldehyde MDA was already expressed at day 0. This is possibly due to the cold storage and transport that is known to lead to glutathione loss (Vreugdenhil et al., 1991). As values of MDA remained stable over the culture period and were not different between systems, we may hypothesize that the presence of glutathione reductase and other antioxidants within the culture medium used (Price et al., 1998) may have played a role and hidden any potential benefit of the PDMS components that shield the tissue fragments from direct contact with ambient oxygen (as a source of reactive oxygen species responsible of oxidative stress).

Our results with the silicone-based MF and AGPC systems are important and encouraging but were modest compared to achievements in IVM of mice ITT. Interestingly, while analyzing our findings, a report on the application of the AGPC system for IVM of rat ITT (Matsumura et al., 2021) also pointed to a limited efficiency of the system in comparison to the previous experiments in mice. In fact, not only was rat spermatogenesis blocked at the round spermatid stage, but

under some oxygen conditions the AGPC system lost its added value (reported in the original studies with mice ITT) in comparison the classical AG approach. One may argue that this could be due to inadequacy of culture media components although in the later study an enriched culture medium was used.

These observations as well as ours, strengthen the fact that culture conditions (e.g., physical conditions i.e., systems, timing, culture medium) for *in vitro* spermatogenesis are not translatable from one species to another. Species-specific culture conditions, notably for humans, should therefore be tailored in the future.

While we consider the results of this work as an added value to the current available literature on IVM of ITT, our comparative study has some limitations that should be taken into consideration. Tissue fragments were heterogeneous in size with smaller sizes in the MF system. This means that smaller fragments could have sustained greater damage during dissection (leading to a poorer outcome in terms of tissue core degeneration with the MF device compared to AGPC) while larger fragments might have suffered from poorer nutrient perfusion.

Culture media perfusion was also different between the static and the MF system and could have interfered with interpretation of some of our results. For example, the testosterone level was low at day 5 in the MF group, most probably not because the LCs were not functional but just because the PVC tubes had to be filled with culture medium prior to the experiment start, leading to a diluted testosterone level in this first recovered supernatant sample (that contains media perfused from day 0 to day 5).

As for germ cell differentiation, while both VASA + SYCP3+ and VASA + CREM + germ cells appeared during culture, their overall numbers were low, and their appearance did not occur in clusters as observed *in vivo*. Reduced efficiency of spermatogenesis *in vitro* is a known phenomenon in mice (Komeya et al., 2019), and an even greater challenge in rats (Reda et al., 2016; Matsumura et al., 2021) or larger mammals (Reda et al., 2016; de Michele et al., 2018; Medrano et al., 2018) and should be the focus of future work in the field of *in vitro* spermatogenesis.

Altogether, we were able to show that the porcine ITT underwent functional maturation, and that spermatogenesis was successfully initiated in tissue fragments (from all piglets) cultured in all four systems *in vitro*. Also, we demonstrated that only the AGPC system outperformed the control (OT), mainly by moderately improving both undifferentiated spermatogonia survival and the efficiency of germ cell differentiation.

It is however important to note that repeat experiments with a higher number of pigs/samples to increase the statistical power might allow to further highlight the differences among all four systems.

We conclude that prior to applying complex organotypic tissue culture systems to the IVM of human ITT, future studies should first focus on improving media components (e.g., growth factors, dosage, timing of administration, etc.) as this seems to be the most important limiting factor for the success of IVM at this stage. It is however not excluded that after an optimal culture medium is established, applying MF and AGPC or other complex culture systems could help in improving the efficiency of spermatogenesis *ex vivo*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MK, FdM, and CW designed the study. MK performed the culture experiments, and the analyses. SVL and TG provided consultancy and supervised the production of the polydimethylsiloxane components for the different systems. CW, JP, FdM, MGG, and MK contributed with data interpretation. CW supervised the project, critically revised the manuscript, and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.884122/full#supplementary-material

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Sertoli and Germ Cells Within Atrophic Seminiferous Tubules of Men With Non-Obstructive Azoospermia

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Background: Infertile men with non-obstructive azoospermia (NOA) have impaired spermatogenesis. Dilated and un-dilated atrophic seminiferous tubules are often present in the testes of these patients, with the highest likelihood of active spermatogenesis in the dilated tubules. Little is known about the un-dilated tubules, which in NOA patients constitute the majority. To advance therapeutic strategies for men with NOA who fail surgical sperm retrieval we aimed to characterize the spermatogonial stem cell microenvironment in atrophic un-dilated tubules.

Methods: Testis biopsies approximately 3x3x3 mm^3 were obtained from un-dilated areas from 34 patients. They were classified as hypospermatogenesis (HS) (n=5), maturation arrest (MA) (n=14), and Sertoli cell only (SCO) (n= 15). Testis samples from five fertile men were included as controls. Biopsies were used for histological analysis, RT-PCR analysis and immunofluorescence of germ and Sertoli cell markers.

Results: Anti-Müllerian hormone mRNA and protein expression was increased in un-dilated tubules in all three NOA subtypes, compared to the control, showing an immature state of Sertoli cells ($p<0.05$). The GDNF mRNA expression was significantly increased in MA ($P=0.0003$). The BMP4 mRNA expression showed a significant increase in HS, MA, and SCO ($P=0.02$, $P=0.0005$, $P=0.02$, respectively). The thickness of the tubule wall was increased 2.2-fold in the SCO-NOA compared to the control ($p<0.05$). In germ cells, we found the DEAD-box helicase 4 (DDX4) and melanoma-associated antigen A4 (MAGE-A4) mRNA and protein expression reduced in NOA (MAGE-A: 46% decrease in HS, 53% decrease in MA, absent in SCO). In HS-NOA, the number of androgen receptor positive Sertoli cells was reduced 30% with a similar pattern in mRNA expression. The γH2AX

expression was increased in SCO as compared to HS and MA. However, none of these differences reached statistical significance probably due to low number of samples.

Conclusions: Sertoli cells were shown to be immature in un-dilated tubules of three NOA subtypes. The increased DNA damage in Sertoli cells and thicker tubule wall in SCO suggested a different mechanism for the absence of spermatogenesis from SCO to HS and MA. These results expand insight into the differences in un-dilated tubules from the different types of NOA patients.

Keywords: non-obstructive azoospermia, un-dilated tubules, hypospermatogenesis, maturation arrest, Sertoli cell only, male infertility

INTRODUCTION

Azoospermia is identified in 1% of all men and up to 20% of infertile men, and is subdivided as obstructive and non-obstructive azoospermia (NOA) (1, 2). NOA, the most severe form of impaired male fertility, is the absence of spermatozoa in the ejaculate caused by reduced or missing spermatogenesis (3). Several potential causes of NOA are known, including varicocele, previous gonadotoxic therapy, Y chromosome microdeletions and Klinefelter's Syndrome, but in up to half of the cases a clear etiology is not identified (4). Based on the histological assessment of testis biopsies, men with NOA, independent of cause, can be classified into one of the three categories: Hypospermatogenesis (HS) - decreased spermatogenesis with all types of germ cells present, maturation arrest (MA) - premature arrest of spermatogenesis, and Sertoli cell-only (SCO) - absence of germ cells (5, 6).

In men diagnosed with NOA, surgical retrieval of spermatozoa, which can be used for intracytoplasmic sperm injection (ICSI), is performed. However, sperm retrieval rates are approximately 50% (7), leaving half of all NOA patients to rely on donor sperm. Novel therapeutic techniques, such as spermatogonial stem cell-based transplantation or autologous tissue transplantation, might represent alternatives to restore fertility in the future (8, 9). In order to advance these strategies more detailed information on the microenvironment of the seminiferous tubules including germ cells and Sertoli cells from different types of NOA patients is required.

Sertoli cells in the seminiferous tubules support the niche in which the spermatogonial stem cells (SSCs) differentiate into spermatozoa (10, 11). A delicate and intricate hormonal balance between Sertoli cell function and SSCs is required to support full spermatogenesis, which also involves Leydig cells providing sufficient amounts of androgens. Testosterone from Leydig cells acts *via* the androgen receptor (AR) which is expressed in mature Sertoli cells but not in the germ cells. Moreover, Anti-Müllerian hormone (AMH) is secreted by immature Sertoli cells and is downregulated by increased intratesticular testosterone during puberty (12). Sertoli cells produce glial cell-derived neurotrophic factor (GDNF) and bone morphogenetic protein 4 (BMP4) that influence undifferentiated spermatogonia in rodent but their role remains unclear in human (13–16). Peritubular myoid cells (PTMCs) constitute the tubular wall and act to contract seminiferous tubules leading to the transfer of immotile sperm. PTMCs exerts paracrine functions on Sertoli cells and Leydig cells (17, 18). Activations of Leydig cell expressed luteinizing hormone(LH) receptor lead to production of testosterone which signal through AR receptors expressed on Sertoli cells (19). Collectively, successful spermatogenesis is dependent on proper function of several cell types in the testis and hormonal stimulation, which in NOA patients are aberrant in one or more, currently unknown, steps.

Men with NOA occasionally have pockets of "dilated" seminiferous tubules. These tubules are likely to have a normal diameter but appear dilated in relation to the surrounding atrophic "un-dilated" tubules. For practical reasons the terms "dilated" and "un-dilated" will be used in the remainder of the text. Dilated tubules often manage, for unknown reasons, to sustain active spermatogenesis while the un-dilated areas lack this ability. Extraction of sperm cells from the dilated tubules by microdissection testicular sperm extraction (mTESE) is used clinically to obtain spermatozoa for ICSI. In an attempt to advance understanding of reasons for the spermatogenic impairment in the un-dilated seminiferous tubules in NOA patients, we explored different markers of germ and Sertoli cells using mRNA expression and immunofluorescence. Thus, in order to understand how to advance spermatogenesis in testis tissue from NOA patients, we aimed at understanding the difference in molecular characteristics of germ cells and Sertoli cells between testes from men with normal spermatogenesis and atrophic un-dilated tubules in testis biopsies from NOA men with HS, MA and SCO.

MATERIALS AND METHODS

Human Testis Materials

Testis tissue was obtained from 34 NOA patients who underwent mTESE as a part of treatment for infertility and from five adult men with normal sperm production and proven fertility, who provided a biopsy in connection with vasectomy. In patients with NOA, testis biopsies approximately 3x3x3 mm^3 were obtained anteriorly right under the tunica albuginea in connection with the mTESE procedure and used for histopathological diagnosis.

Additional testis biopsies that did not show dilated seminiferous tubules (as observed under the operating microscope) were taken from the same area. Consequently, in NOA men, only biopsies from un-dilated tubules were used in this study. All testicular biopsies for research purposes were placed immediately in McCoy 5A medium (modified 22330-021, Gibco, UK) for transportation to the Laboratory of Reproductive Biology for the cryopreservation. Then they were equilibrated for 20 min in media consisting of 1.5 M ethylene glycol, 0.1 M sucrose, 10 mg/ml human serum albumin (HSA) (CSL Behring, Germany), frozen and cryopreserved in -196°C liquid nitrogen according to previous published methods (20).

Clinical Workup

All men with NOA were diagnosed after a complete medical history and physical examination including scrotal ultrasound. Azoospermia was diagnosed according to the 5th edition of World Health Organization (WHO) laboratory manual for the "Examination and processing of human semen" (21). A full hormonal evaluation including serum levels of follicle-stimulating hormone (FSH), LH, inhibin. B and testosterone was performed. All men were assessed for the presence of Y chromosome microdeletions and a karyotype was obtained. Fasting morning blood samples were drawn. Serum testosterone levels were analyzed by a chemiluminescence immunoassay (Access 2, Beckman Coulter, Brea, CA, USA), follicle-stimulating hormone FSH and LH by a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland), and inhibin B by a specific two-sided enzyme-immunometric assay (Inhibin B gen II, Beckman Coulter Ltd, High Wycombe, UK). Culture of peripheral blood lymphocytes was used for karyotype analysis. The diagnosis of NOA was made after a complete assessment by an experienced andrologist using all the above information. Men with testis size larger than 15ml, indication of obstructive causes of azoospermia and Klinefelter's Syndrome were not included.

Tissue Processing and Histology

Thawing was done by progressively using the following three thawing media. Thawing medium I: 0.75 M ethylene glycol, 0.25 M sucrose in PBS, and 10 mg/ml HSA; thawing medium II: 0.25 M sucrose in PBS, and 10 mg/ml HSA; thawing medium III: PBS and 10 mg/ml HSA, each medium for 10 min (20). After thawing, one testis biopsy from each patient was divided into three parts. One part for immunostaining, one for qPCR, and one was re-frozen for future use. Tissues for immunostaining were fixed in 4% paraformaldehyde (PFA) at room temperature overnight, embedded in paraffin and cut in 5-μm sections. Sections were deparaffinized in xylene, rehydrated with series of graded ethanol. Sections for histological evaluation were stained with periodic acid-Schiff reagent (PAS). Due to the heterogeneity of testis tissues in men with NOA, the spermatogenetic status of all 34 samples was histologically re-analyzed on sections stained with PAS in addition to the original histopathological diagnosis made as part of clinical care using a 3x3x3 mm^3 biopsy taken anteriorly under the tunica albuginea. If different, the histopathological diagnosis from un-dilated tubules was used.

Immunofluorescence Staining

After deparaffinization and rehydration of the section, antigens were retrieved by boiling in TEG buffer (10 mM Tris, 0.5 mM ethylene glycol-bis (2-aminoethylehter)-N, N, N', N'-tetraacetic acid (EGTA), pH 9) for 30 min. After non-specific binding was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer (50 mM Tris, 150 mM NaCl, pH7.6) for 30 min, the sections were incubated with primary antibodies at +4°C overnight. All antibodies were diluted in TBS with 1% BSA. The primary antibodies included (**Supplementary Table 1**): a monoclonal mouse anti-melanoma antigen genes-A (MAGE-A) (1:100) for detection of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12 which were previously shown to be present in spermatogonia and some spermatocytes (22, 23), a monoclonal mouse anti-ubiquitin carboxyl-terminal hydrolase L1 (UCHL1, also known as protein gene product 9.5, PGP 9.5) (1:100) present in human spermatogonia (24), a polyclonal rabbit anti- phosphorylation of histone H2AX in Serine 139 (γH2AX) (meiotic marker in germ cells and a marker of DNA double-strand breaks in somatic cells) (1:1500) (25, 26), a monoclonal mouse anti-Vimentin (1:200) for somatic cells (27), a polyclonal rabbit anti-SOX9 (1:100) for Sertoli cells (28), a polyclonal goat anti-Müllerian hormone (AMH) (1:100) for immature Sertoli cells (28), a monoclonal rabbit anti-androgen receptor (AR) (1:100) for mature Sertoli cells (29), a rabbit polyclonal anti-alpha-smooth muscle actin (ACTA) (1:150) for peritubular myoid cells (30), a goat polyclonal anti-cytochrome P450 17A1 (CYP17A1) (1:200) for Leydig cells (23, 31). And universal negative control serum (NC498H, Biocare Medical) was used for negative control. After washing 3 times in TBS with Tween 20®, the slides were incubated with the following secondary antibodies at room temperature for 1h: FITC-conjugated donkey anti-mouse IgG antibody/Alexa Fluor 594 donkey anti-rabbit IgG antibody/ Alexa Fluor 568 donkey anti-goat IgG antibody (1:500, Jackson ImmunoResearch). After washing, the slides were stained with 4',6 – diamidino-2-phenylindole (DAPI) for nuclear staining. Pictures were taken on a Zeiss Axiophot microscope, operated with a Leica DFC420C digital microscope camera and LAS V4.9 software (Leica).

Five seminiferous tubules per section were randomly chosen (two tubules from upper panel, two tubules from lower panel, one tubule from the center) and two histological sections at different depths of the biopsy were evaluated per sample. The number of SOX9/AR-positive Sertoli cells and MAGE-A-positive germ cells per square millimeter (mm^2) was calculated. Firstly, we measured the diameter of the tubule to calculate the area (mm^2) of the tubule. Then, we counted the number of SOX9/AR/MAGE-A-positive cells within each tubule. We got the number of SOX9/AR/MAGE-A-positive cells/tubule area (mm^2). Finally, we calculated the mean number of SOX9/AR/MAGE-A-positive cells/mm^2 based on 10 tubules per testis biopsy. For the MAGE-

A-positive germ cell, we only counted positive cells located on the basement membrane to exclude counting of spermatocytes. We evaluated AMH expression based on its staining intensity from "strong" "moderate" "mild" to "absent". The thickness of tubule wall was measured at four points around the circumference of each tubule, with ACTA-positive signal thickness measured at the ends of two perpendicular axes.

RNA Extraction and Quantitative RT-PCR

RNA was extracted from testis biopsies from all 34 NOA patients and five normal control testis tissues using Trizol reagent (Invitrogen) and 1-bromo-3-chloropropane (Sigma). Then the following steps were performed using the RNeasy Kit (Qiagen) according to the manufacturer's protocol. The average RNA obtained was 22ug with a range from 5ug to 80ug. The 260/280 ratio was found to be 2.1 ± 0.04 (range: 2.00 to 2.14). The total RNA from each sample used to make the cDNA was 1ug. cDNA was synthesized by using of High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The dilution of the cDNA template used to do qPCR was 1:5.

Quantitative RT-PCR was performed by using TaqMan Fast Advanced Master Mix (Applied Biosystems) and the LightCycler 480 Instrument II (Roche Diagnostics). The cDNA from each sample was used as a RT-PCR template to be detected of the expression of those genes from TaqMan primer assays (**Supplementary Table 2**). GAPDH was used as an internal control and the normal adult testis tissue as a positive control.

Statistics

Individual values were presented as mean ± standard deviation (sd). GraphPad Prism version 8.0 was used for statistical analyses. Kruskal-Wallis test with Dunn's multiple comparisons test was used to analyze the difference of mRNA expression among HS, MA, SCO to the normal control group, respectively. For the histological results, each subtype of NOA was compared to the normal control. The statistical difference of the number of MAGE-A/SOX9/AR-positive cells and the thickness of tubule wall among HS, MA, SCO to the normal control group was analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Chi-square test was used to analyze the percentage of AMH-positive tubules in NOA subtypes compared to normal control group. Correlations between mRNA expression and hormone levels were tested by Spearman rank correlation coefficient. P values < 0.05 were considered statistically significant.

RESULTS

PAS Staining for Histopathological Diagnosis

Based on histological evaluation of biopsies from the un-dilated areas of seminiferous tubules and the clinical diagnosis, our material consisted of 5 HS, 14 MA, and 15 SCO samples (**Table 1**).

Characterization of Niche Related Cells in the Testis From Different Types of NOA Patients Using Immunofluorescence Staining

Somatic Cells

SOX9-positive Sertoli cells appeared disorganized with a scattered distribution in HS while organized with a more circle like location close to the basal membrane in MA compared to normal group, contrasting the SCO samples which resembled the normal tissue with cells tidily located close to the basement membrane and near one another to each other (**Figures 1A, B**). The total number of SOX9-positive cells was counted in ten tubules and the average number per tubule was 20 in HS, 19 in MA, 24 in SCO, and 18 in normal control group. The average number of AR-positive cells per tubule was 21 in HS, 22 in MA, 24 in SCO, and 28 in normal control group. Combining the different size of tubules, the number of SOX9/AR-positive Sertoli cells per mm^2 was not significantly different in NOA subtypes compared to the normal group (**Figures 1A-D**). The percentage of AMH-positive tubules showed significant increase in HS, MA, and SCO compared to normal control group (HS, MA, SCO: $P<0.001$). The percentage of tubules with "strong" AMH expression in each NOA subtypes was significantly increased to normal control (HS: $P<0.001$, MA: $P=0.001$, SCO: $P<0.001$). The same significant increase was observed in tubules with "moderate" AMH expression (HS, MA, SCO: $P<0.001$). The percentage of tubules with "mild" AMH expression significantly increased only in SCO ($P=0.006$) (**Figures 1E, F**).

Visualizing the peritubular myoid cells (PTMCs) *via* ACTA-positive staining, the distribution of cells and the thickness of tubule wall appeared to be normal in both HS and MA patients, while the tubule wall in the SCO patient was 2.2-fold thicker than in the normal testis tissues ($P = 0.001$) (**Figures 2A, B**).

Leydig cells visualized *via* CYP17A1 expression showed no difference in distribution between any of the NOA samples and the normal controls (**Figure 3**). Due to the tiny testis biopsies the interstitial tissues were not fixed well and quantification of the CYP17A1-positive Leydig cells was not done in this study.

TABLE 1 | Age and etiology of non-obstructive azoospermia (n = 34) and normal control.

Variable	HS (n = 5)	MA (n = 14)	SCO (n = 15)	Normal control (n = 5)
Age years, mean (sd)	33 (3)	32 (6)	35 (6)	32 (5)
Cryptorchidism N (%)	2 (40.0)	5 (35.7)	4 (26.7)	–
Varicocele N (%)	3 (60.0)	5 (35.7)	5 (33.3)	–
Idiopathic N (%)	–	3 (21.4)	5 (33.3)	–
AZFc N (%)	–	1 (7.1)	–	–
Cancer treatment N (%)	–	–	1 (6.7)[a]	–

[a]*This patient had a 46, X, inv (Y) (p11.2 q11.22) karyotype, all other included NOA patients all had normal karyotype.*

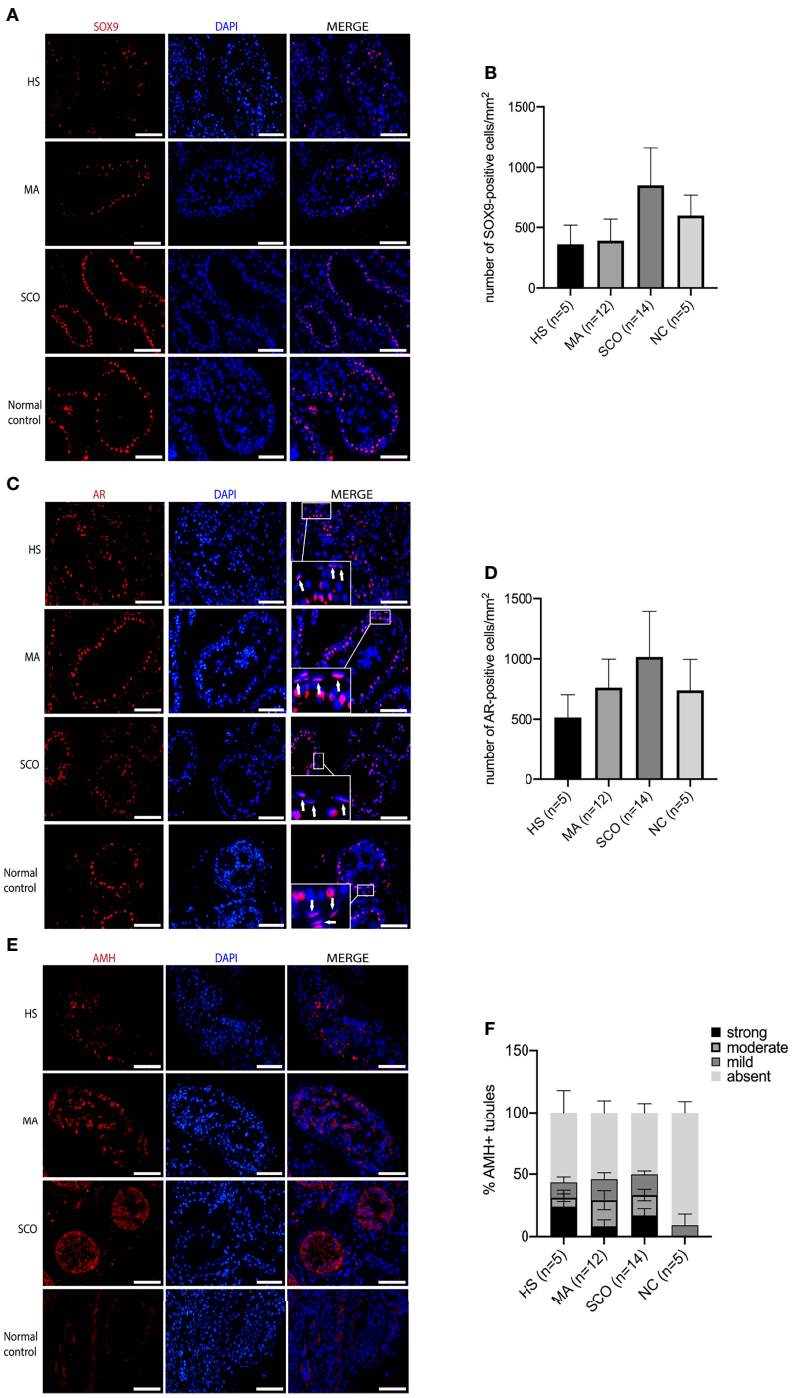

FIGURE 1 | Immunofluorescence staining of Sertoli cell markers in un-dilated seminiferous tubules from hypospermatogenesis (HS), maturation arrest (MA), Sertoli cell only (SCO), and normal control (NC) samples. **(A)** Sox9 (red) for Sertoli cells. **(B)** The number of Sox9-positive cells per mm^2 was counted from ten tubules of each biopsy. **(C)** AR (red) for mature Sertoli cells, white arrow indicated that AR also expressed in PTMCs. **(D)** AR-positive cells per mm^2 was counted from ten tubules of each biopsy. **(E)** AMH (red) for immature Sertoli cells. **(F)** AMH staining was categorized into "strong" "moderate" "mild" "absent" based on staining intensity. The percentage of AMH-positive tubules (including strong, moderate, mild expression of AMH) showed significant increase in HS, MA, and SCO comparing to normal control group. The percentage of tubules with "strong" AMH expression in each NOA subtypes was significantly increased to normal control (HS: $P < 0.001$, MA: $P = 0.001$, SCO: $P < 0.001$). The percentage of tubules with "moderate" AMH expression in each NOA subtypes was also significantly increased (HS, MA, SCO: $P< 0.001$). The percentage of "mild" AMH expression only significantly increased in SCO ($P = 0.006$). DAPI (blue) for nuclear staining. Scale bar: 100 μm. n represent the number of individuals included.

γH2AX-Positive Cells

In HS, there was a strong homogenous nuclear staining and prominent γH2AX foci in most of the germ cells (**Figure 4**). Some nuclear staining was weak and dispersed, but most were dotted and strong. Almost no γH2AX expression was detected in Sertoli cells stained with Vimentin. In MA, γH2AX was strongly expressed in the nuclei of germ cells in a dotted and dispersed pattern. There were a few Sertoli cells with γH2AX staining (**Figure 4**). In SCO, almost all Sertoli cells showed a pronounced expression γH2AX in the nuclei (**Figure 4**).

Germ Cells

Both germ cell markers, MAGE-A and UCHL1, were positively expressed in the un-dilated seminiferous tubules of HS and MA patients indicating the presence of germ cells (**Figures 5A, B**). The average number of MAGE-A-positive cells per tubule was 17 in HS, 15 in MA, 0 in SCO, and 26 in normal control group. The number of MAGE-A-positive cells per mm^2 was not significantly different in HS and MA compared to the normal group (**Figure 5C**). In contrast, no expression of MAGE-A and UCHL1 was present in SCO patients (**Figures 5A-C**). The staining patterns in these three types were mainly cytoplasmic and the location of positive cells was near the basement membrane of the seminiferous tubules as observed in the normal control.

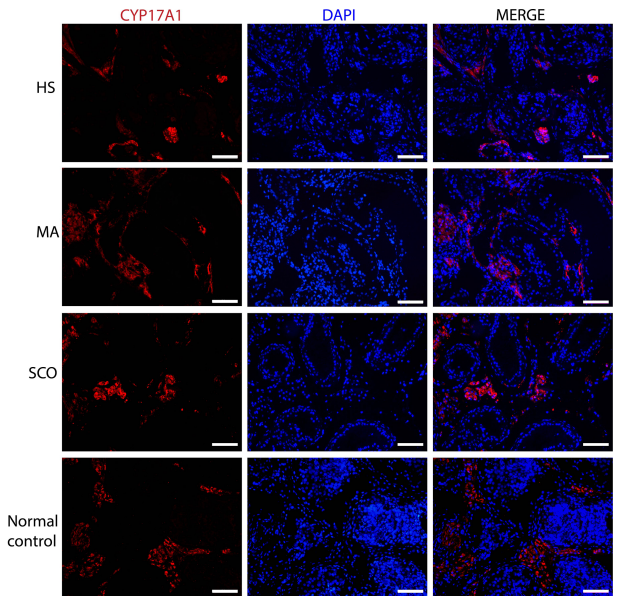

FIGURE 3 | Immunofluorescence staining of Leydig cell marker in un-dilated seminiferous tubules from HS, MA, SCO, and NC samples. CYP17A1 (red) for Leydig cells, DAPI (blue) for nuclear staining. Scale bar: 100 μm.

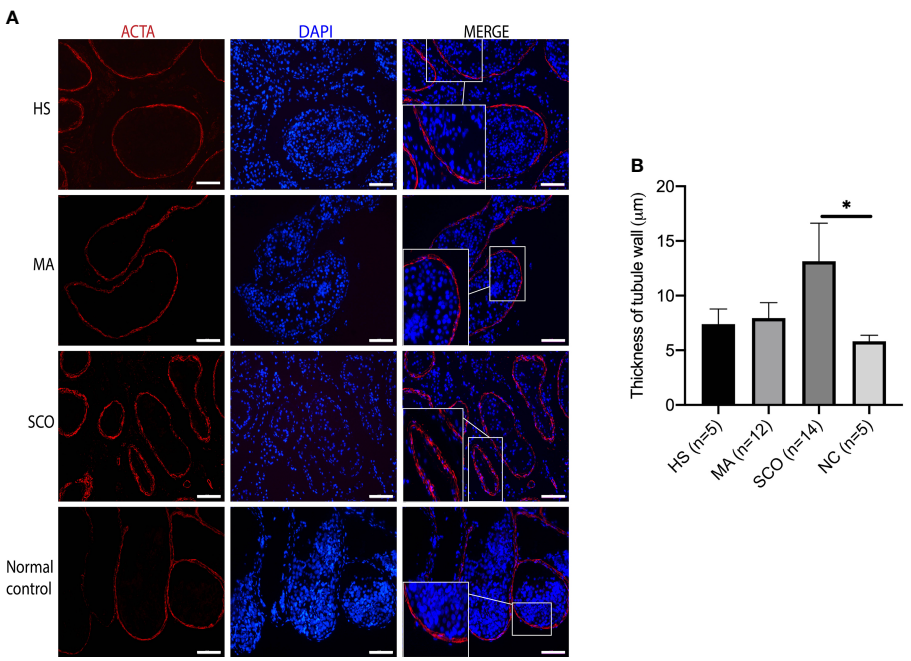

FIGURE 2 | Immunofluorescence staining of peritubular myoid cell (PTMC) marker in un-dilated seminiferous tubules from HS, MA, SCO, and NC samples. **(A)** Alpha-smooth muscle actin (ACTA) (red) for PTMC, DAPI (blue) for nuclear staining, Scale bar: 100 μm. **(B)** Thickness analysis of tubule wall. Asterisk indicated significant difference between SCO and normal control group (*$p < 0.05$).

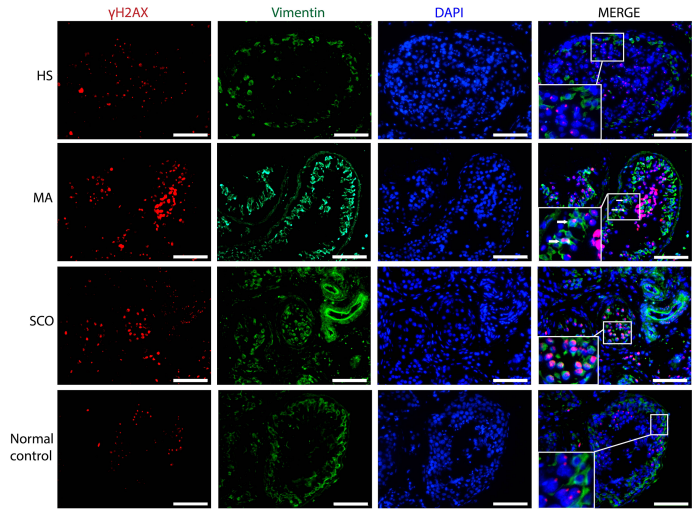

FIGURE 4 | Immunofluorescence staining of γH2AX (red), somatic cell marker Vimentin (green), nuclear marker DAPI (blue) in un-dilated seminiferous tubules in samples from HS, MA, and SCO samples. White arrow indicated γH2AX-positive Sertoli cells in MA. As almost all Sertoli cells expressed γH2AX in SCO, we did not add arrow to indicate them. There were no γH2AX-positive Sertoli cells in HS and normal control group. Scale bar: 100 μm.

qPCR Analysis of Niche Related Cells

The mRNA expression of DEAD-box helicase 4 (*DDX4*, also named *VASA*) germ cell-specific gene was significantly decreased in HS, MA, SCO compared to normal control group (P=0.006, P=0.004, P=0.004, respectively) (**Figure 6**). In SCO, the mRNA expression of *MAGE-A4* was significantly downregulated (P=0.004) (**Figure 6**). The mRNA expression of *AMH* (indicating immature Sertoli cells) was significantly increased in MA and SCO compared to normal control group (P=0.001, P =0.0002, respectively) (**Figure 6**). Contrary to the *AMH*, the mRNA expression of androgen receptor (*AR*) (indicating mature Sertoli cell) showed no difference in the three types of NOA samples compared to the normal group (**Figure 6**). The mRNA expression of GDNF was significantly increased in MA (P=0.0003) (**Figure 6**). The mRNA expression of BMP4 showed a significant increase in HS, MA, and SCO (P=0.02, P=0.0005, P=0.02, respectively) (**Figure 6**).

Correlation Between mRNA Expression and Serum FSH

In MA, serum FSH showed a significant negative correlation with mRNA expression of *CYP17A1* (r=-0.55, P=0.04) (**Table 2**). No other correlations were found between the remaining genes and hormone values.

DISCUSSION

This study demonstrated pronounced testicular differences within the un-dilated seminiferous tubules between different types of NOA patients and normal control group. The maturation state of Sertoli cells, the number of germ cells, and the thickness of tubule wall were distinct in NOA subtypes. Collectively, the microenvironments within the un-dilated tubules are different in different subtypes of NOA patients.

The maturation state of Sertoli cells was evaluated by AMH and AR expression on both mRNA and protein level. The higher *AMH* mRNA expression and more tubules with AMH expression in all three NOA subtypes compared to that in the normal group suggests that there were more immature Sertoli cells within the un-dilated tubules from all three NOA subtypes. Earlier studies also showed that immature Sertoli cells were observed in the testis from infertile adult men (32–34). After a higher dilution of AMH antibody employed, we found that there was a threshold level of detection and variable AMH expression in the normal control group. The results are consistent with a previous report that showed both AMH positive staining within the seminiferous tubules of patients with Sertoli-cell-only syndrome (SCOS) and in men with normal spermatogenesis, but staining intensity was stronger in SCOS than in normal group (28). Furthermore, in adult men, AMH is secreted in both serum and seminal plasma (35). It was reported that the seminal AMH concentration was variable ranging from undetectable to a high level (36) suggesting a Sertoli cell secretion. Collectively, this argues for a mild AMH expression in tubules from normal fertile men. In HS and MA, the number of AR-positive mature Sertoli cells appeared to be reduced. This tendency was also shown in *AR* mRNA expression level. In SCO, the number of SOX9- and AR-positive cells was slightly higher than the normal group, but no significant differences were found, and the *AR* mRNA expression showed a decreased tendency. Thus, there are both mature and immature Sertoli cells in SCO patients, to what extend both were increased needs to be addressed in a future study. The increased expression of AMH in Sertoli cells may reflect a maturation failure of Sertoli cells in connection with puberty (37, 38) or alternatively de-differentiation of mature

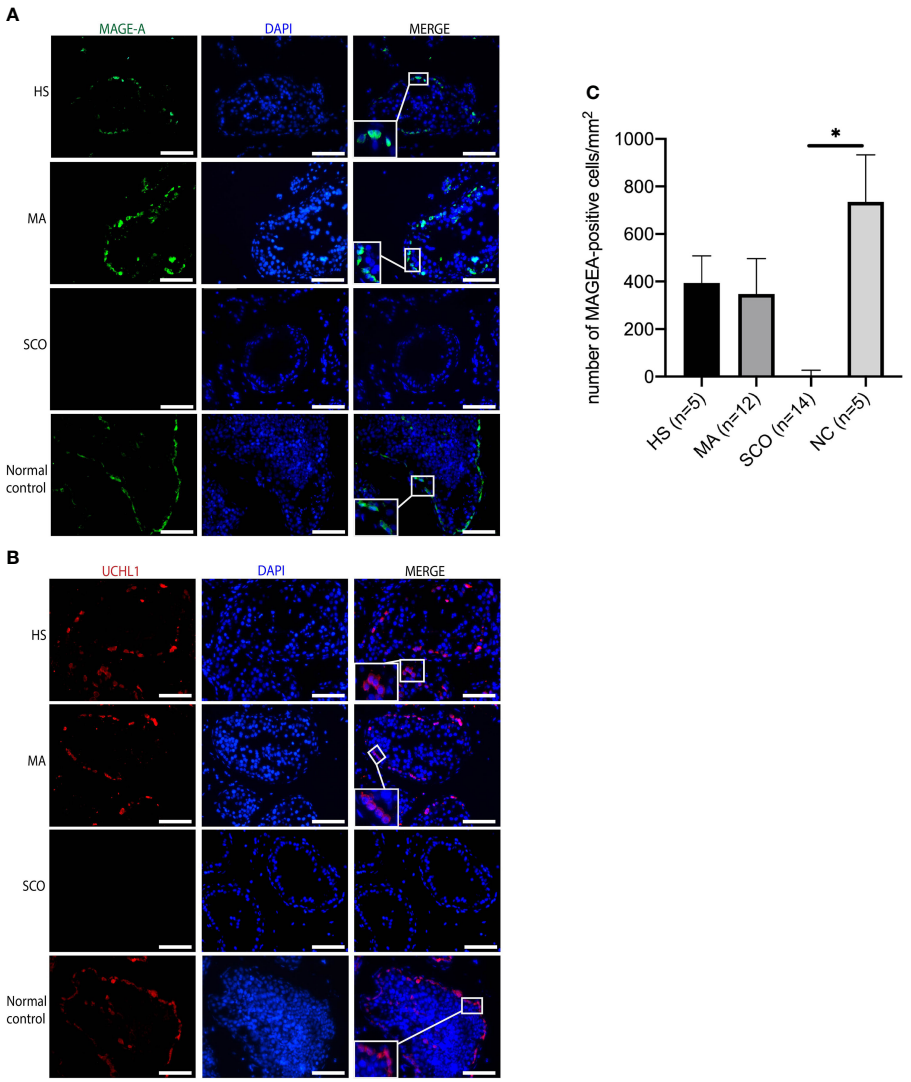

FIGURE 5 | Immunofluorescence staining of germ cell markers in un-dilated seminiferous tubules from HS, MA, SCO, and NC samples. **(A)** MAGE-A (green) for germ cells, **(B)** UCHL1 (red) for germ cells, DAPI (blue) for nuclear staining, Scale bar: 100 µm. **(C)** The number of MAGE-A-positive cells per mm^2 was counted based on ten tubules of each biopsy. Asterisk indicated significant difference between SCO and normal control group (*$p < 0.05$).

Sertoli cells to acquire a more immature state (34). However, the current study is unable to distinguish between maturation failure and de-differentiation of the Sertoli cells.

It has been reported in mice that the overexpression of GDNF showed accumulation of undifferentiated spermatogonia (14) and inhibiting GDNF signaling could promote differentiation of SSC (39). We found GDNF mRNA overexpressing in MA suggesting GDNF could contribute to the maturation failure in MA. It has been reported that FSH induces the GDNF expression (40) and the higher FSH values (i.e. 20.6 IU/L in MA NOA subgroup as compared to 1.5-12.4 IU/L in the normal group) could explain the observed higher expression of GDNF. All three subtypes of NOA showed overexpression of BMP4 mRNA confirming a previous study that showed BMP4 overexpression in MA-NOA and SCO-NOA at the protein level in relation to control group (41). In contrast, mRNA expression of BMP4 was reported lower in SCO (42). This discrepancy may be due to mixing the control group with both tissues from men with hypospermatogenesis and normal spermatogenesis.

The germ cell status was evaluated by using qPCR and immunofluorescence. The mRNA expression of *DDX4* and *MAGE-A4* was significantly reduced in SCO and exhibited a decreased tendency in HS and MA. This is consistent with other studies that reported a reduced germ-cell niche in HS and MA from infertile men (43–45). The number of MAGE-A-positive germ cells was slightly reduced in HS and MA while absent in SCO. Similarly, the germ cell specific UCHL1 expression was absent in SCO. The attenuated germ cell numbers may either be related to meiotic defects (46, 47) and/or impairment and immaturity of Sertoli cells being unable to support full germ cell maturation.

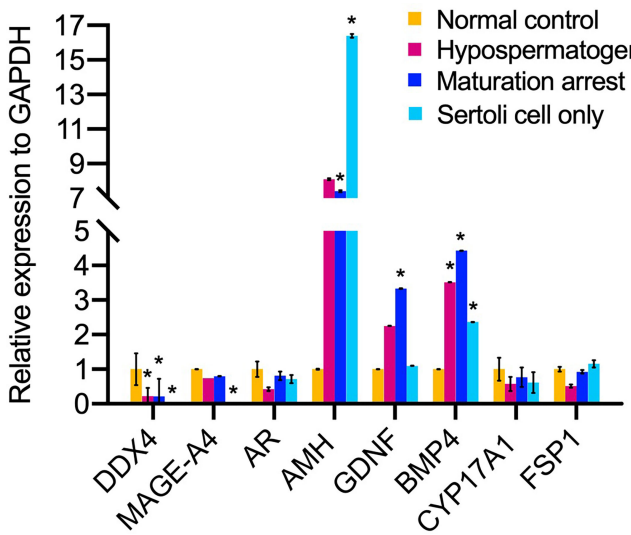

FIGURE 6 | The mRNA expression analysis of niche related cells in un-dilated seminiferous tubules from non-obstructive azoospermia patients (NOA) and healthy adult men. The relative mRNA expression of germ cell *DDX4*, *MAGE-A4*, and somatic cell *AMH*, *AR*, *GDNF, BMP4*, *CYP17A1*, fibroblast-specific protein 1 (*FSP1*) to internal control *GAPDH*. Kruskal-Wallis test, *$p < 0.05$.

The presence of histone H2AX phosphorylation (γH2AX) was used to identify germ cells in the prophase of the first meiotic division (48), but Sertoli cells expressing DNA damage response also become stained (25). H2AX is a histone variant that belongs to the H2A family and prevent genome instability and cancer (49–51), while the phosphorylated form γH2AX is regarded as a robust marker of DNA double-strand breaks (DSBs) (52, 53). We found that the γH2AX expression was different between three types of NOA patients. There were more Sertoli cells expressing γH2AX in SCO than in HS and MA biopsies. The

TABLE 2 | Correlation between mRNA expression and clinical hormones.

Variable	AR	AMH	HS (n = 5) GDNF	BMP4	FSH	Inhibin B
VASA	-0.30,.68	0.40,.52	0.20,.78	-0.10,.95	0.10,.95	0.50,.45
MAGE-A4	-0.30,.68	-0.90,.08	-0.70,.23	0.00, >0.99	-0.10,.95	-0.10,.95
CYP17A1	-0.50,.45	-0.10,.95	-0.70,.23	-0.90,.08	0.10,.95	-0.40,.52
FSP	0.10,.95	0.30,.68	0.10,.95	-0.70,.23	0.70,.23	-0.20,.78
LH	0.67,.27	0.67,.27	0.36,.63	-0.56,.37	0.36,.63	-0.87,.07
T	0.60,.35	0.80,.13	0.40,.52	-0.50,.45	0.20,.78	-0.80,.13
Variable	**AR**	**AMH**	**MA (n=14) GDNF**	**BMP4**	**FSH**	**Inhibin B**
VASA	-0.02,.95	0.02,.93	0.22,.46	0.20,.48	0.41,.15	-0.08,.80
MAGE-A4	-0.29,.33	0.09,.76	0.18,.56	0.01,.99	0.49,.09	-0.27,.36
CYP17A1	0.45,.11	-0.13,.65	0.09,.75	0.13,.67	-0.55,.04	0.26,.36
FSP	0.52,.06	0.03,.92	0.28,.34	0.20,.50	-0.41,.14	0.45,.11
LH	-0.25,.38	0.17,.55	-0.46,.10	-0.34,.23	0.58,.03	-0.68,.01
T	0.36,.20	0.02,.94	0.28,.32	0.29,.32	-0.11,.71	0.15, 61
Variable	**AR**	**AMH**	**SCO (n=15) GDNF**	**BMP4**	**FSH**	**Inhibin B**
VASA	-0.29,.29	-0.18,.52	0.35,.30	0.23,.50	-0.28,.30	-0.07,.80
MAGE-A4	0.02,.95	-0.02,.95	-0.40,.75	0.60,.42	-0.23,.41	-0.32,.24
CYP17A1	-0.19,.49	0.01,.96	0.12,.68	0.31,.25	-0.36,.19	0.30,.28
FSP	0.03,.91	-0.10,.71	0.13,.65	0.08,.78	-0.06,.82	0.18,.51
LH	0.25,.37	0.20,.47	-0.13,.64	-0.09,.75	0.66,.01	-0.31,.27
T	-0.21,.45	0.07,.80	0.15,.59	-0.07,.81	0.40,.14	0.31,.27

Values are r (correlation coefficient), P value. P <.05 was considered significant by Spearman's rank correlation.

accumulation of γH2AX positive staining in Sertoli cells demonstrated that the Sertoli cells may be undergoing accelerated degradation.

The present study suggested that Sertoli cells in un-dilated seminiferous tubules of NOA patients were immature and expressed an increased DNA damage compared to normal controls. In MA, few Sertoli cells appeared to have DNA damages response. In SCO, many γH2AX-positive Sertoli cells may undergo DNA damage response.

Collectively, the present study suggests that un-dilated seminiferous tubules from three subtypes of NOA patients show a different expression of cell specific markers that most likely reflect their compromised ability to sustain spermatogenesis or alternatively that compromised germ cells influence Sertoli cell function. However, both HS and MA subtype of NOA patients demonstrate, in un-dilated seminiferous tubules, the quantitative presence of germ cell numbers approaches that of normal testis in some instances. It may therefore be envisioned that these germ cells could be matured to haploid germ cells and used in connection with ART. This will require the development of an *in vitro* culture system providing a proper environment, for instance by co-culture with spent media from cultures of mature normal Sertoli cells. Alternatively, testis tissue from men with NOA could be cultured together with mature Sertoli cells from a normal testis without direct cell contact between the NOA tissue and the supplied normal Sertoli cells. Alternatively, or in combination, growth factors and hormones known to advance meiosis may be used to advance meiosis in cultures of testis tissue from NOA patients in whom sperm retrieval was unsuccessful (54, 55). The fact that some of these men with NOA actually present with a few dilated areas of seminiferous tubules with spermatogenesis suggests that it is possible to define conditions of sufficient quality to advance meiosis to the haploid state.

The thicker tubule wall of seminiferous tubules in NOA patients with SCO may affect the contractility of the tubules and the propulsion of the tubular contents to the rete testis.

A limitation of our study is the relatively small sample size. More samples are necessary in the future for further exploration of mechanisms behind NOA.

In conclusion, this study provides insights into understanding the un-dilated (atrophic) tubules which constitute a major part of seminiferous tubules of NOA patients. Improvement of Sertoli cell function either during *in vitro* culture or by co-culture with Sertoli cells from fertile men may constitute strategies for fertility restoration in patients with different types of NOA that fail surgical sperm retrieval. The impairment and immaturity of Sertoli cells and germ-cell loss are likely to contribute to the impaired spermatogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethical committee of the Capital Region of Denmark (H-16033784) and the Region of Southern Denmark (S-20200088). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CJ, DW, LM, AG, NJ, MF, DO, LD, JF, CA, JS conceived and designed the experiments. CJ collected testis biopsies for experimental use. DW performed the experiments. CJ, DW, LM, SH, SP, EN, CA performed data analysis and interpretation. DW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.825904/full#supplementary-material

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Clinical Outcomes and Live Birth Rate Resulted From Microdissection Testicular Sperm Extraction With ICSI-IVF in Non-Obstructive Azoospermia: A Single-Center Cohort Study

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Background: Most of data available in the literature reported the sperm retrieval rate and limited intracytoplasmic sperm injection (ICSI) results of microdissection testicular sperm extraction (micro-TESE) in non-obstructive azoospermia (NOA) patients with different etiologies. Unfortunately, there is currently a lack of comprehensive data to guide clinicians in conducting comprehensive consultations with NOA patients.

Objectives: To obtain more comprehensive evidence-based data and clinical outcomes for better consultation of NOA patients who opted to undergo micro-TESE combined with ICSI-IVF

Methods: It was a retrospective study involved 968 NOA patients underwent micro-TESE during January 2015 to December 2019. Embryological, clinical, and live birth outcomes were demonstrated comprehensively and three kinds of stratification analyses were performed based on ICSI-IVF cycles using frozen and fresh sperm, different etiologies of NOA and various amounts of sperm retrieved.

Results: The sperm retrieval rate was 44.6%, and ICSI was performed in 299 couples leading to 150 clinical pregnancies and 140 live-birth deliveries. The clinical pregnancy rate (CPR) was 50.17%, and the cumulative live birth rate (LBR) was 46.82%, and the low birth defects rate was 1.43%. No significant difference was observed about cumulative LBR in frozen sperm group and fresh sperm group (47.5% vs 42.9%, $P>0.05$). NOA patients with

AZFc microdeletions had the lowest rate of a high-score embryo on day 3 (4.4%, $P<0.05$) and the lowest cumulative LBR (19.4%, $P<0.05$). NOA patients with lower sperm count (having fewer than 20 sperms retrieved) had significantly lower cumulative LBR than those with higher sperm count (having more than 20 sperms retrieved) (28.1% vs 51.9%, $P<0.05$).

Conclusions: For those NOA patients who stepped in ICSI-IVF cycles, the cumulative LBR was 46.82%. No significant difference was indicated in the LBR between ICSI-IVF cycles using frozen or fresh testicular sperm. Compared to other etiologies, NOA caused by AZFc microdeletions have the poorest embryological and clinical outcomes. Patients with less testicular sperm retrieved have poorer embryological and clinical outcomes.

Keywords: intracytoplasmic sperm injection, microdissection testicular sperm extraction, non-obstructive azoospermia, testicular sperm, pregnancy rate, live birth rate

1 INTRODUCTION

Non-obstructive azoospermia (NOA) is the most severe form of male infertility and is characterized by the testis's inability to produce mature sperm, and NOA accounts for 60% of all patients with azoospermia (1). Based on the different causes of non-obstructive azoospermia, non-genetic etiologies include cryptorchidism, heat exposure, infections, and chemoradiotherapy. The most common genetic causes are Y chromosome microdeletions and chromosomal abnormalities (2). However, most NOA patients have the unknown cause of their azoospermia (3). Because of spermatogenesis, couples with NOA used donor sperm or opted for adoption to have children before current sperm retrieval methods (4).

The development of intracytoplasmic sperm injection (ICSI) in 1992 provided a novel opportunity for azoospermia patients to become fathers (5). Sperm obtained through testicular sperm extraction (TESE) was first performed on NOA patients for ICSI in 1995 (6). TESE-ICSI then became a routine procedure to treat NOA patients. Studies reported that the sperm retrieval rate (SRR) of TESE in the NOA population was approximately 30 - 50%. However, these studies' selective bias makes numbers controversial (7, 8). Subsequently, various surgical techniques, such as multiple testicular biopsies and fine-needle aspiration, were used to improve SRR and reduce complications in NOA patients, but each has its limitations (9). In the past 20 years, microdissection testicular sperm extraction (micro-TESE) has gradually become a popular surgical technique with a high SRR and low tissue loss (10, 11). However, most of the previous studies were focus on SRR, and few could provide comprehensive follow-up data on ICSI-IVF outcomes of NOA patients, explicitly focusing on the use of fresh or frozen sperm, cumulative pregnancy rate (PR), and live birth rate (LBR), not alone birth defect rate (12, 13).

Our goal was to obtain more comprehensive evidence-based data and clinical outcomes for better preoperative consultation and counseling of NOA patients who opted to undergo micro-TESE combined with ICSI-IVF. This retrospective study aimed to analyze the effects of frozen or fresh sperm, different etiologies, and retrieved sperm quantity on ICSI-IVF outcomes after micro-TESE.

2 MATERIALS AND METHODS

2.1 Patients Selection

This retrospective study included 968 men with NOA who underwent micro-TESE in the Reproductive Medicine Center of Third Affiliated Hospital of Guangzhou Medical University between January 2015 and December 2019 (**Figure 1**). All semen samples were centrifuged at 3,000 g for 30 minutes to confirm azoospermia and on at least three separate occasions. NOA patients underwent a complete clinical evaluation to determine the etiology of azoospermia, including clinical history, physical examination, testicular ultrasound, evaluation of sex hormone levels [follicle stimulating hormone (FSH), luteinizing hormone (LH) testosterone (T), estradiol (E2), and prolactin (PRL)], karyotyping and Y chromosome microdeletion analysis.

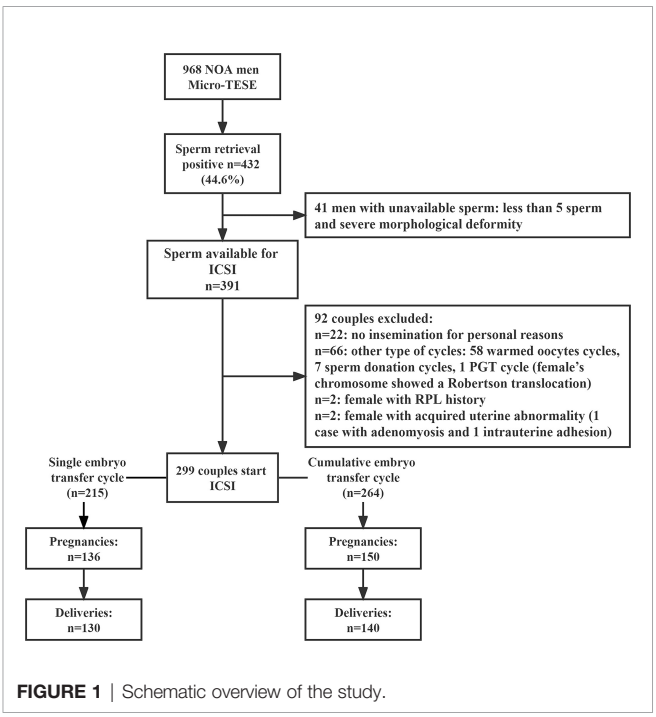

FIGURE 1 | Schematic overview of the study.

If azoospermia combined with testicular atrophy and abnormality of sex hormone, the etiological diagnosis process was as follows: ①NOA caused by Klinefelter's syndrome or AZF microdeletions was diagnosed if there was karyotyping or Y chromosome microdeletion abnormalities. ②NOA caused by orchitis was diagnosed if there was a history of orchitis after mumps infection in adolescence or adulthood. ③NOA caused by cryptorchidism was diagnosed if there was a combination of ultrasound diagnosis or previous history of cryptorchidism (especially surgical history). ④Idiopathic NOA was diagnosed by excluding known causes of NOA. When testicular volume, texture and endocrine examination were normal, testicular pathological biopsy was required to confirm whether there was spermatogenesis disorder. All patients underwent micro-TESE were routinely performed with testicular biopsy during surgery to further confirm spermatogenesis dysfunction. Patients with a history of micro-TESE, a history of radiation and chemotherapy, anejaculation, or hypogonadotropic hypogonadism were excluded. The same surgeon (GA) performed all micro-TESE procedures. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (reference number 2017-055) and was carried out in accordance with the Helsinki Declaration. Due to the retrospective nature, informed consent was not required, and patients` data were used anonymously.

Before micro-TESE surgery was conducted, complete clinical consulting was performed regarding the treatment protocol selection and male factors that may influence the outcome of ICSI-IVF treatment. In addition, the female's ovarian reserve would be assessed in detail before the eventual decision on the conduction of micro-TESE surgery to reduce the risk of cycle cancellation due to the female factors. Advanced age (≥40 years old, 6 cases), diminished ovarian reserve (AMH ≤ 1ng/ml), or poor ovarian response (≤4 oocytes retrieved) were all defined as risk factors for adverse outcomes, and patients would be informed of high risk of ICSI-IVF failure.

All NOA couples were offered the option to receive fresh or frozen testicular sperm for ICSI cycles before surgical sperm retrieval and be informed of their respective advantages and disadvantages. For those using frozen sperm, controlled ovarian stimulation (COS) will be initiated after the sperm is successfully assessed for cryopreservation. For those using fresh sperm, they would be informed that using fresh sperm can minimize the operation step of sperm before injection which may reduce energy loss and the risk of operation error, especially when very few sperm are retrieved. However, the simultaneous ICSI cycles of fresh sperm and egg collection may result in unnecessary COS, oocytes retrieval, and passive egg freezing due to sperm retrieval failure or unimplemented surgery.

2.2 Micro-TESE

A single surgeon (GA) performed the micro-TESE surgical procedures as described in the previous literature (14), a few minor and technical modifications, including the use of saline instead of Ringer's solution as the rinsing fluid and suturing the albuginea testis with a 5-0 polypropylene suture instead of 6-0. Instead of the surgeon grinding the testicular tissue on the operating table, an embryologist grind the testicular tissue and microscopically search sperm on a bench setting near the operating table (15). If no sperm was identified in one testis, micro-TESE of the contralateral testis was performed immediately.

2.3 Evaluation of Sperm Quality

After testicular tissue was retrieved from the testis and transferred to embryologist, spermatozoa was further processed to be released. Procedures were performed at room temperature as follows: wash testicular tissue with 1 ml G-MOPS-Plus fluid (Vitrolife, Sweden) to remove the red blood cells; Move testicular tissue into a new petri dish and add 1 ml new G-MOPS-Plus fluid and grind the tissue into tiny patches by using microscopic forceps to release spermatozoa. Then, the grinded testicular tissue homogenate was evaluated under high power magnification (200×).

All sperm were counted out and recorded if the amount was less than 100. When the total sperm amount was more than 100, it will be recorded as ">100". We defined the lower sperm count (LSC) group as having fewer than 20 sperms and defined the higher sperm count (HSC) group as more than 20 sperms. Motile spermatozoa rate was determined (motile spermatozoa rate = motile sperm/total sperm × 100%). If the total number of sperm was greater than 100, the number of motile sperm was quantified in 100 sperm. The sperm deformity was not be record because it could not be assessed accurately under 200× magnification.

For statistical analysis, there was another method for the evaluation of sperm amount before and after cryopreservation (as section 4 below).

2.4 Testicular Tissue Suspension Cryopreservation and Thawed

After testicular tissue homogenate was evaluated and transferred to the embryology laboratory, a freezing process was immediately performed. First, transfer all fluid and tissues into a 15 ml centrifuge tube; Add 1 ml fresh G-MOPS-Plus fluid to the petri dish to wash additional tissue from the dish and transfer into the 15-ml tube; Incubate at room temperature for five minutes; Aspirate the supernatant to a fresh new 15-ml centrifuge tube; Add 1ml G-MOPS-Plus fluid to the pellet, and transfer the supernatant to the fresh new 15-ml tube. Centrifuge the tube at 400g for 10 minutes, and remove all the supernatant (remaining 0.5ml). Sucking 50μl out to count under the microscope and recorded the amount of sperm in each high magnification field, and recycle these 50μl. Next, the freezing procedures were performed as follows: add freezing medium (Test York Buffer with Gentamicin Sulfate) (Irvine Scientific, USA) and G-MOPS-Plus in a 1:1 ratio into a pellet and resuspend, aliquot resuspended pellet into cryovials; and place at t 4°C for 30 minutes; Suspend the mixture prior to placing in liquid nitrogen for 1 hour. Transfer the cryovials into a liquid nitrogen container for long-term cryopreservation. The thawing process was performed as follows. Remove the cryovial from the liquid nitrogen container, place it at room temperature for 10 minutes; Transfer the thawed liquid to a fresh 15ml centrifuge

tube; Add drops of IM washing buffer (Vitrolife, Sweden) to the tube, and mix gently. Centrifuge at 400g for 10 minutes, and then remove the supernatant. Resuspend the pellet with 1-2 ml IM washing buffer. After the second wash, resuspend the sperm pellet with 50-100 μl G-MOPS-Plus fluid. The count and motility of thawed sperm were reassessed.

2.5 Ovarian Stimulation and Oocytes Retrieval

In couples who had testicular sperm was retrieved and frozen, or in couples undergoing a synchronous micro-TESE-ICSI procedure, female partners underwent ovarian stimulation using recombinant FSH or hMG combined GnRH antagonists or GnRH-a (16). Oocyte–cumulus complexes were recovered 36h after administering 5000 or 10 000 IU of hCG.

2.6 ICSI Procedure, Embryo Culture, and Transfer

For couples who had sperm cryopreserved, sperm were thawed only when females had oocytes retrieved. For couples who underwent synchronous micro-TESE-ICSI treatment, oocytes were vitrified if no sperm retrieved. Next, ICSI-IVF and fertilization assessment were performed as previously described by Liu (16). Fertilization rates were expressed as the percentage of oocytes with two distinct pronuclei per injected metaphase II oocytes. Embryos were scored by their morphological appearance according to the Society for Assisted Reproductive Technology scoring system (17). Normally cleaving embryos with ≥5 cells and ≤20% fragmentation were considered eligible for transfer. Up to two embryos were transferred into the uterine cavity on day 3 (preferred) or day 5 after injection. The remaining embryos were frozen directly for the next thawed transfer cycles. All patients had completed at least one embryo transfer by the end of follow-up.

2.7 Pregnancy Follow-Up

Pregnancy was diagnosed by elevated serum hCG levels (≥25IU/L) 14 days after embryo transfer. Clinical pregnancy was defined as a visible gestational sac at transvaginal ultrasound 4-5 weeks after embryo transfer. Pregnancy loss was defined as the loss of a clinical pregnancy before 28 weeks of gestation. Live birth was defined as the birth of at least one living child, irrespective of gestation duration. The cumulative pregnancy or live birth was defined as clinical pregnancy or at least one live-born baby resulting from an ICSI-IVF initiated cycle.

2.8 Statistical Analysis

Statistical analyses were performed with SPSS statistical software for Windows, version 22.0 (SPSS, Chicago, IL, USA). All data were normally distributed and continuous variables were expressed as mean ± SD. The 'Student's t-test was used for comparison of continuous variables. One-way ANOVA was used to assess outcomes among more than two groups. Chi-squared ($\chi 2$) or Fisher's exact test was used for proportions. Differences were considered statistically significant when the p-value was <0.05.

3 RESULTS

3.1 Micro-TESE and Sperm Recovery

A total of 968 patients with NOA underwent micro-TESE, and 432 had sperm retrieved (sperm retrieval rate, SRR=44.6%). A total of 299 patients were with sperm retrieved were consecutively enrolled in ICSI treatment (**Figure 1**). The SRR showed significantly different among the different etiologies, including orchitis (81.2%), Klinefelter Syndrome (KS) (43.6%), Y chromosome azoospermia factor c (AZFc) microdeletions (68.6%), cryptorchidism (62.4%), and idiopathic (31.1%) (**Supplementary Table 1**) (p<0.01).

Sperm quality and quantity were evaluated under a microscope before freezing and after thawing, and assessment included a total of sperm count, percentage of motile sperm, and the percentage of abnormal sperm (teratozoospermia). No significant differences were found in the total sperm count and motility of testicular sperm between the different groups (**Supplementary Table 2**) (p>0.05).

3.2 Outcomes of ICSI-IVF

ICSI-IVF was performed in 299 couples, leading to 136 clinical pregnancies and 130 live-birth deliveries in the first embryo transfer cycle, and 150 clinical pregnancies and 140 live-birth deliveries in cumulative embryo transfer cycle (**Figure 1**).

Among those NOA patients with micro-TESE-ICSI, the fertilization rate was 69.85%, the 2PN rate was 61.49%, the day 3 utilization rate was 28.35%, the rate of high-score embryo on day 3 was 12.75%, and the rate of unavailable embryos was 11.7%. In the first embryo transfer cycle, the clinical pregnancy rate (CPR) was 45.48% and the LBR was 43.49%. In the cumulative embryo transfer cycle, CPR was as high as 56.82% and LBR was 53.03% in those with available embryos, and 50.17% of CPR and 46.82% of LBR in those undergoing ICSI cycles, and 34.72% of CPR and 32.41% in those who have successful sperm retrieval (**Table 1**, **2**).

According to the follow-up data, there were 26 premature infants in 140 newborns and two cases of birth defects recorded. One case is a cardiovascular malformation, and the other is a cleft lip and palate. (**Table 2**).

First of all, a stratification analysis based on using frozen or fresh testicular sperm was performed. All couples undergoing the ICSI-IVF cycle were divided into frozen sperm group and fresh sperm group (257 cases with frozen sperm *vs.* 42 cases with fresh sperm). The fertilization rate, 2 primary nucleus (PN) rate, numbers of available and high-score embryos on day 3 were comparable between these two groups. There were no significant differences in PR and LBR between these two groups no matter in the first embryo transfer cycle (PR: 45.9% vs. 42.9%, LBR: 44.0% vs. 40.5%, p>0.05) or cumulative embryo transfer cycles (PR: 51.0% vs. 45.2%, LBR: 47.5 vs. 42.9%, p>0.05). Besides, the percentage of patients with no available embryo was low in both frozen and fresh sperm groups and was not significantly different between the two groups (11.3% vs. 14.3%). Follow-up data showed that compared to the fresh sperm group, singleton newborns of frozen sperm group have higher height (49.84 ± 2.04cm *vs.* 48.50 ± 3.03cm, p<0.05) (**Table 3**).

TABLE 1 | Basic characteristics and ICSI outcomes of the NOA patients with sperm retrieved by Micro-TESE.

Variable	Parameters
Male	
Age (yr.)	31.52 ± 4.77
BMI (kg/m^2)	24.49 ± 3.46
Left Testicular volume (ml)	7.01 ± 3.77
Right Testicular volume (ml)	6.97 ± 3.82
Hormone profile	
FSH (IU/L)	19.42 ± 10.89
LH (IU/L)	9.46 ± 5.58
T (ng/ml)	10.73 ± 6.92
Female	
Age (yr.)	29.10 ± 4.05
AMH (ng/ml)	5.02 ± 3.83
BMI (kg/m^2)	21.94 ± 3.39
Infertility type	
Primary (%)	83.9 (251/299)
Secondary (%)	16.1 (48/299)
COS-ICSI outcomes	
Days of ovarian stimulation	10.61 ± 2.20
Total gonadotropin dose (IU)	1888.93 ± 920.67
Oestradiol level on hCG trigger day (pmol/L)	11139.50 ± 4606.68
Progesterone level on hCG trigger day (nmol/L)	2.41 ± 1.33
The endometrial thickness on hCG trigger day (mm)	10.52 ± 2.02
Number of oocytes retrieved	14.38 ± 7.24
Number of MII oocytes for ICSI	10.96 ± 7.97
Fertilization rate (%)	69.85 (2289/3277)
2PN rate (%)	61.49 (2015/3277)
2PN cleavage rate (%)	81.30 (1861/2289)
Day 3 utilization rate of MII eggs for ICSI (%)[1]	28.35 (929/3277)
Rate of high-score embryo on day 3 (%)[2]	12.75 (257/2015)
No. of cycles without available embryos/ICSI cycles (%)	11.7 (35/299)
Number of available embryos on day 3	3.11 ± 2.80
Number of high-score embryos on day 3	0.86 ± 1.14

Data are expressed as mean ± SD unless indicated otherwise.

[1]Computational formula: number of available embryos on day3/number of MII oocytes for ICSI.

[2]Computational formula: number of high-score embryos on day 3/number of 2PN.

ICSI, intracytoplasmic sperm injection; NOA, non-obstructive azoospermia; Micro-TESE, microdissection testicular sperm extraction; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; AMH, anti-mullerian hormone; COS, controlled ovarian stimulation; hCG, human choroinic gonadotrophin; PN, Primary nucleus.

Next, a stratification analysis based on different etiologies of NOA was performed. The results showed a statistical difference in day 3 utilization rate of MII eggs for ICSI among each group, with the lowest percentage observed in patients with Y chromosome AZFc microdeletions (22.3%), followed by KS (24.4%), cryptorchidism (27.2%), orchitis (30.8%), and was observed to be the highest in patients with idiopathic NOA (31.7%). The percentage of patients with no available embryo was significantly different among the five etiologies of NOA, with the highest percentage in patients with Y chromosome AZFc microdeletions (25.8%) (**Supplementary Figure 1**). Besides, the lowest rate of the high-score embryo on day 3 (4.4%), lowest cumulative CPR (22.6%), lowest cumulative LBR with successful sperm retrieval (19.4%), and the highest rate of premature birth (50%) were observed in patients with Y chromosome AZFc microdeletions ($p<0.05$) (**Table 4**).

Furthermore, a stratification analysis based on sperm quantity was performed. All patients who successfully entered the ICSI cycle were separated into lower sperm count (LSC, ≤20 sperms) and higher sperm count (HSC, >20 sperms) groups. No significant difference was observed in male or female age between the two groups ($p>0.05$). NOA patients with LSC showed higher FSH levels, and their female partners underwent more partial oocyte freezing (23.4%, $p<0.05$). In all initial ICSI cycles (including cycles without available embryos on day 3) in the PP set, couples with LSC had significantly lower 2PN cleavage rate, lower day 3 utilization rate of MII eggs for ICSI, a lower rate of the high-score embryo on day 3, lower cumulative CPR (29.7% vs. 55.7%, $p<0.05$), lower cumulative LBR (28.1% vs. 51.9%, $p<0.05$) and a significantly higher risk of no available embryos on day 3 compared with couples with HSC ($p<0.05$). Additionally, significantly lower cumulative CPR (37.5% vs. 56.5%, $p<0.05$) was observed in couples with LSC than couples with HSC when patients who had no available embryos were excluded (**Table 5**).

4 DISCUSSION

This study's main strength is comprehensively analyzing clinical outcomes for NOA patients undergoing micro-TESE and ICSI-IVF cycle which minimally or barely reported in the past

TABLE 2 | Embryo transfer and live birth outcomes.

Variable	NOA couples
1st embryo transfer cycle	
No. of embryos transferred	1.55 ± 0.50
Clinical pregnancy rate (%)	45.48 (136/299)
Live birth rate (%)	43.49 (130/299)
Cumulative Pregnancy	
No. of patients with clinical pregnancy/undergoing ICSI cycles with available embryos (%)	56.82 (150/264)
No. of patients with clinical pregnancy/undergoing ICSI cycles (%)	50.17 (150/299)
No. of patients with clinical pregnancy/successful sperm retrieval (%)	34.72 (150/432)
Cumulative Live birth	
No. live birth/clinical pregnancy (%)	93.33 (140/150)
Singleton (%)	87.14 (122/140)
Twins (%)	12.86 (18/140)
No. live birth/undergoing ICSI cycles with available embryos (%)	53.03 (140/264)
No. live birth/undergoing ICSI cycles (%)	46.82 (140/299)
No. live birth/successful sperm retrieval (%)	32.41 (140/432)
No. premature infants/live birth (%)	18.57 (26/140)
No. cesarean delivery/live birth (%)	31.43 (44/140)
No. birth defects/live birth (%)[#]	1.43 (2/140)
Gender	
Male (%)	39.24 (62/158)
Female (%)	60.76 (96/158)
Birth weight (g)	2981.27 ± 534.65
Singleton (n =122)	3174.18 ± 419.13
Twins (n =18, 36 babies)	2327.50 ± 324.95
Height (cm)	48.65 ± 3.13
Singleton (n =122)	49.66 ± 2.23
Twins (n =18, 36 babies)	45.19 ± 3.32

Data are expressed as mean ± SD unless indicated otherwise.
[#]One case is a cardiovascular malformation, and the other is a cleft lip and palate.
ICSI, intracytoplasmic sperm injection; NOA, non-obstructive azoospermia.

literature. A total of 299 NOA patients who had stepped in ICSI cycles were evaluated, which is the largest retrospective study with sample size at present. In addition, female factors that may affect the ICSI-IVF outcomes were excluded, including recurrent pregnancy loss, abnormal karyotype, known uterine anomalies, and adenomyosis, improving extrapolation of our results.

4.1 Micro-TESE-ICSI has Considerable Live Birth Outcomes

According to our study, the fertilization rate was as high as 69.85%, the day 3 utilization rate of MII eggs for ICSI was 28.35%, the cumulative CPR was 50.17%, and the cumulative LBR was 46.82% which high up to the same as conventional ICSI-IVF as reported (18). Besides, there were no stillbirths, pregnancy complications, or neonatal complications in our study. Overall, the opportunity for a NOA patient to be a biological father is 14.5% (140/968) for NOA patients before he accept micro-TESE treatment. With successful sperm retrieving, this opportunity rise up to 32.41%, and once his sperm be assessed as suitable for ICSI cycle, the chance rise up to 46.82%. It is worth to mention that only about 11.7% patients may not be able to obtain available embryos according to one ICSI cycle. However, poor outcomes were observed in this study in those couples who has female risk factors (with advanced female age ≥40 years old; AMH ≤ 1ng/ml; ≤4 oocytes retrieved) for ICSI-IVF. All of these patients treated with ICSI lead to one live-birth delivery in 6 patients aged ≥40 years, three live-birth deliveries in 16 diminished ovarian reserve patients, and 15 live-birth deliveries in 29 poor ovarian response patients (data not included in the results section). The female-factor combined with male-factor assessment refined our clinical consultation on ICSI treatment for couples with NOA. Those detailed data will help physicians provide with sufficient counseling and avoid patients` over-high expectations of treatment. Thus, this study provides a tremendous clinical basis for reproductive medicine specialists to better understand the clinical outcome of micro-TESE combined with ICSI-IVF cycle, which allows for more comprehensive preoperative clinical consultation and preparations.

4.2 Similar Live Birth Outcomes Were Observed in ICSI-IVF Cycles Using Frozen or Fresh Testicular Sperm

Not all NOA patients have access to fresh sperm, and couples who want to synchronize the ICSI-IVF cycles with fresh sperm and oocyte have to face the risk of oocyte freezing and sperm donation-IVF. Therefore, sperm cryopreservation before ICSI may be more reasonable and reduce unnecessary risks for females. Cryopreservation of testicular sperm has long been used in assisted reproductive technology (19). Previous studies

TABLE 3 | Comparison of the ICSI outcomes between frozen and fresh sperm.

	Frozen sperm (n=257)	Fresh sperm (n=42)	*P*-value
Age (yr)			
Male	31.58 ± 4.91	31.10 ± 3.86	0.540
Female	29.01 ± 4.07	29.64 ± 3.94	0.350
COS-ICSI outcomes	/	/	/
Days of ovarian stimulation	10.62 ± 2.29	10.50 ± 1.55	0.738
Total gonadotropin dose (IU)	1914.55 ± 959.68	1732.14 ± 617.26	0.235
Estradiol level on HCG trigger day (pmol/L)	10789.34 ± 4528.86	13282.10 ± 4550.46	0.001*
Progesterone level on HCG trigger day (nmol/L)	2.35 ± 1.30	2.77 ± 1.50	0.059
The endometrial thickness on HCG trigger day (mm)	10.56 ± 2.08	10.29 ± 1.66	0.417
Number of oocytes retrieved	13.87 ± 7.13	17.50 ± 7.23	0.002*
Number of MII oocytes for ICSI	11.02 ± 8.29	10.62 ± 5.73	0.766
Fertilization rate (%)	70.1 (1985/2831)	68.2 (304/446)	0.403
			0.516#
2PN rate (%)	61.8 (1750/2831)	59.4 (265/446)	0.333
			0.684#
2PN cleavage rate (%)	91.9 (1609/1750)	95.1 (252/265)	0.072
			0.447#
Number of available embryos on day 3	3.09 ± 2.94	2.86 ± 2.25	0.625
			0.074#
Number of high-score embryos on day 3	0.84 ± 1.15	0.98 ± 1.05	0.474
			0.899#
Day 3 utilization rate of MII eggs for ICSI (%)[1]	28.7 (812/2831)	26.2 (117/446)	0.286
			0.899#
Rate of high-score embryo on day 3 (%)[2]	12.3 (216/1750)	15.5 (41/265)	0.16
			0.062#
No. of cycles without available embryos/ICSI cycles (%)	11.3 (29/257)	14.3 (6/42)	0.575
1st embryo transfer cycle			
No. of embryos transferred	1.56 ± 0.51	1.61 ± 0.55	0.533
Clinical pregnancy rate (%)	45.9 (118/257)	42.9 (18/42)	0.712
			0.452#
Live birth rate (%)	44.0 (113/257)	40.5 (17/42)	0.672
			0.499#
One ICSI cycle			
Cumulative pregnancy rate per women (%)	51.0 (131/257)	45.2 (19/42)	0.491
Cumulative live birth rate per women (%)	47.5 (122/257)	42.9 (18/42)	0.579
No. live birth/clinical pregnancy (%)	93.1 (122/131)	94.7 (18/19)	0.793
Singleton (%)	86.9 (106/122)	88.9 (16/18)	0.813
Twins (%)	13.1 (16/122)	11.1 (2/18)	
Birth defect (%)	1.6 (2/122)	0 (0/18)	0.584
Gender	/	/	0.941
Male (%)	39.1 (54/138)	40.0 (8/20)	/
Female (%)	60.9 (84/138)	60.0 (12/20)	/
Birth weight (g)			
Singleton	3194.67 ± 405.16	3038.44 ± 495.32	0.166
Twins	2302.50 ± 331.11	2527.50 ± 197.55	0.196
Height (cm)			
Singleton	49.84 ± 2.04	48.50 ± 3.03	0.024*
Twins	44.91 ± 3.40	47.50 ± 1.00	0.143

Data are expressed as mean ± SD unless indicated otherwise

1 Computational formula: number of available embryos on day3 / number of MII oocytes for ICSI.

2 Computational formula: number of high-score embryos on day 3 / number of 2PN.

**Significantly different.*

 # P value after correcting the estradiol level on HCG trigger day.

ICSI, intracytoplasmic sperm injection; COS, controlled ovarian stimulation.

P value after corrected the estradiol level on HCG trigger day.

confirmed significant differences between fresh and frozen sperm by ejaculation, regardless of total sperm count, motility, or morphology of sperm (20); however, the differences contributed little to the outcome of ICSI cycles (21). Due to controversial evaluations with NOA patients, ICSI-IVF outcomes are controversial because of different cryopreservation methods (12, 22).

Our study reported an improved laboratory technique of testicular tissue suspension cryopreservation in NOA patients after sperm retrieval from micro-TESE. We established a technique and method to assess the quantity and quality of sperm, which is simple, reliable, and can be easily used as a conventional assessment method, even for too extremely few spermatozoa patients. We found no difference in the average

TABLE 4 | Comparison of the ICSI outcomes of NOA patients with different etiologies.

	Idiopathic (n=112)	Orchitis (n=37)	Klinefelter`s Syndrome (n=80)	AZFc Microdeletions (n=31)	Cryptorchidism (n=39)	*P*-value
Male`s age (yr.)	32.48 ± 5.45	32.22 ± 4.42	30.05 ± 4.22	31.03 ± 3.93	31.46 ± 4.01	0.009*
Female`s age (yr.)	29.50 ± 4.06	30.68 ± 4.30	27.94 ± 4.16	28.42 ± 3.16	29.38 ± 3.60	0.006*
Male's FSH (IU/L)	16.14 ± 10.16	22.16 ± 10.56	24.00 ± 11.29	19.72 ± 9.46	16.59 ± 9.76	0.000*
No. of MII oocytes for ICSI	11.79 ± 10.98	11.95 ± 6.91	10.69 ± 5.45	8.97 ± 4.78	9.79 ± 3.71	0.329
No. of available embryos on day3	3.73 ± 3.50	3.68 ± 3.00	2.61 ± 1.85	2.00 ± 2.07	2.67 ± 1.88	0.003*
No. of high-score embryos on day 3	1.06 ± 1.36	1.05 ± 1.15	0.76 ± 0.93	0.26 ± 0.51	0.77 ± 0.99	0.006*
Day 3 utilization rate of MII eggs for ICSI %[1]	31.7 (418/1320)	30.8 (136/442)	24.4 (209/855)	22.3 (62/278)	27.2 (104/382)	0.000*
Rate of high-score embryo on day 3%[2]	14.9 (119/801)	14.7 (39/266)	11.7 (61/522)	4.4 (8/180)	12.2 (30/246)	0.003*
No. of cycles without available embryos/ICSI cycles (%)	13.4 (15/112)	5.4 (2/37)	8.8 (7/80)	25.8 (8/31)	7.7 (3/39)	0.062
Sperm quantity (%)	/	/	/	/	/	0.011*
≤20 sperms	15.2 (17/112)	13.5 (5/37)	31.2 (25/80)	35.5 (11/31)	15.4 (6/39)	/
>20 sperms	84.8 (95/112)	86.5 (32/37)	68.8 (55/80)	64.5 (20/31)	84.6 (33/39)	/
Sperm motility rate (%, mean ± SD)	13.69 ± 9.96	11.46 ± 9.93	12.53 ± 11.23	10.23 ± 9.88	16.07 ± 10.71	0.118
Pregnancy	/	/	/	/	/	/
No. of patients with clinical pregnancy/undergoing ICSI cycles with available embryos (%)	58.8 (57/97)	62.9 (22/35)	57.5 (42/73)	30.4 (7/23)	61.1 (22/36)	0.113
No. of patients with clinical pregnancy/undergoing ICSI cycles (%)	50.9 (57/112)	59.5 (22/37)	52.5 (42/80)	22.6 (7/31)	56.4 (22/39)	0.021*
No. of patients with clinical pregnancy/successful sperm retrieval (%)	42.0 (68/162)	63.8 (30/47)	46.6 (54/116)	14.3 (7/49)	46.6 (27/58)	0.000*
Live birth	/	/	/	/	/	/
No. live birth/clinical pregnancy (%)	91.2 (52/57)	95.5 (21/22)	95.2 (40/42)	85.7 (6/7)	95.5 (21/22)	0.805
Singleton (%)	86.5 (45/52)	90.5 (19/21)	92.5 (37/40)	66.7 (4/6)	81.0 (17/21)	0.378
Twins (%)	13.5 (7/52)	9.5 (2/21)	7.5 (3/40)	33.3 (2/6)	19.0 (4/21)	0.095
No. live birth/undergoing ICSI cycles with available embryos (%)	53.6 (52/97)	60.0 (21/35)	54.8 (40/73)	26.1 (6/23)	58.3 (21/36)	
No. live birth/undergoing ICSI cycles (%)	46.4 (52/112)	56.8 (21/37)	50.0 (40/80)	19.4 (6/31)	53.8 (21/39)	0.018*
No. live birth/successful sperm retrieval (%)	37.0 (60/162)	57.4 (27/47)	44.8 (52/116)	12.2 (6/49)	44.8 (26/58)	0.000*
No. premature infants/live birth (%)	15.4 (8/52)	14.3 (3/21)	25.0 (10/40)	50.0 (3/6)	9.5 (2/21)	0.150
No. cesarean delivery/live birth (%)	36.5 (19/52)	42.9 (9/21)	22.5 (9/40)	33.3 (2/6)	23.8 (5/21)	0.412
No. birth defects/live birth (%)	1.9 (1/52)	0	2.5 (1/40)	0	0	0.892
Gender	/	/	/	/	/	0.906
Male (%)	42.4 (25/59)	34.8 (8/23)	34.9 (15/43)	37.5 (3/8)	44.0 (11/25)	/
Female (%)	57.6 (34/59)	65.2 (15/23)	65.1 (28/43)	62.5 (5/8)	56.0 (14/25)	/
Birth weighBirth weight (g)	/	/	/	/	/	/
Singleton (n =122)	3254.44 ± 396.96	3178.68 ± 398.13	3063.51 ± 478.25	2995.00 ± 528.93	3239.71 ± 301.16	0.252
Twins (n =18, 36 babies)	2156.43 ± 419.94	2495.00 ± 82.26	2459.17 ± 265.87	2411.25 ± 87.21	2402.50 ± 209.61	0.152
Height (cm)	/	/	/	/	/	/
Singleton (n =122)	49.84 ± 2.04	49.53 ± 2.82	49.41 ± 2.42	48.00 ± 2.45	50.29 ± 1.21	0.352
Twins (n =18, 36 babies)	44.36 ± 3.78	48.00 ± 3.16	45.83 ± 3.37	44.25 ± 0.96	45.25 ± 3.01	0.381

Data are expressed as mean ± SD unless indicated otherwise.

*[1] and [2], the definitions see **Table 1**.*

** Significantly different.*

TABLE 5 | Comparison of the ICSI outcomes of NOA patients with different sperm quantity.

	Lower sperm count(n =64)	Higher sperm count(n =235)	*P*-value
Male's age (y)	31.11 ± 4.56	31.63 ± 4.83	0.444
Female's age (y)	28.97 ± 4.30	29.14 ± 3.99	0.770
Male's FSH (IU/L)	21.89 ± 12.62	18.75 ± 10.29	0.041*
No. of MII oocytes for ICSI	15.09 ± 6.76	14.19 ± 7.37	0.376
No. of available embryos on day3	2.14 ± 1.92	3.37 ± 2.94	0.002*
No. of high-score embryos on day 3	0.58 ± 0.87	0.94 ± 1.19	0.025*
Day 3 utilization rate of MII eggs for ICSI %[1]	9.67 ± 5.05	11.31 ± 8.58	0.145
Rate of high-score embryo on day 3%[2]	9.5 (37/390)	13.5 (220/1625)	0.031*
No. of partial oocytes freezing cycles/OPU cycles (%)	23.4 (15/64)	11.9 (28/235)	0.020*
No. of patients without available embryos/ICSI cycles (%)	25.0 (16/64)	8.1 (19/235)	0.000*
Pregnancy			
No. of patients with clinical pregnancy/undergoing ICSI cycles with available embryos (%)	39.6 (19/48)	60.6 (131/216)	0.008*
No. of patients with clinical pregnancy/undergoing ICSI cycles (%)	29.7 (19/64)	55.7 (131/235)	0.000*
No. of patients with clinical pregnancy/successful sperm retrieval (%)	19.0 (24/126)	52.9 (162/306)	0.000*
Live birth			
No. live birth/clinical pregnancy (%)	94.7 (18/19)	93.1 (122/131)	0.793
Singleton (%)	100.0 (18/18)	85.2 (104/122)	0.081
Twins (%)	0 (0/18)	14.8 (18/122)	
No. live birth/undergoing ICSI cycles with available embryos (%)	37.5 (18/48)	56.5 (122/216)	0.017*
No. live birth/undergoing ICSI cycles (%)	28.1 (18/64)	51.9 (122/235)	0.001*
No. live birth/successful sperm retrieval (%)	17.5 (22/126)	48.7 (149/306)	0.000*
No. premature infants/live birth (%)	5.6 (1/18)	22.1 (27/122)	0.101
No. cesarean delivery/live birth (%)	22.2 (4/18)	32.8 (40/122)	0.367
No. birth defects/live birth (%)	5.6 (1/18)	0.8 (1/122)	0.114
Gender	/	/	0.974
Male (%)	38.9(7/18)	39.3(55/140)	/
Female (%)	61.1(11/18)	60.7(85/140)	/
Birth weight (g)	/	/	/
Singleton (n =122)	3073.33 ± 461.11	3191.63 ± 411.33	0.271
Twins (n =18, 36 babies)	0	2327.50 ± 324.95	/
Height (cm)	/	/	/
Singleton (n =122)	48.94 ± 2.82	49.79 ± 2.10	0.139
Twins (n =18, 36 babies)	0	45.19 ± 3.32	/

Data are expressed as mean ± SD unless indicated otherwise; [1] and [2], the definitions see ***Table 1****.*
** Significantly different.*

number and the percentage of motile spermatozoa between frozen-thawed sperm and fresh sperm. At the same time, frozen-thawed sperm with ICSI resulted in a similar fertilization rate (70.1% vs. 68.2%, p=0.403) and day 3 utilization rate of oocytes (28.7% vs. 26.2%, p=0.286) compared with fresh sperm.

According to previous researches, the clinical pregnancy rate between fresh sperm and frozen sperm remains controversial. Park reported that patients with frozen spermatozoa had significantly higher pregnancy and implantation rates than fresh sperm (23). Madureira showed that the fertilization rate and clinical pregnancy rate were higher in fresh sperm from non-mosaic KS patients by TESE (24). A systematic review and meta-analysis revealed that in men with NOA showed that the ICSI-IVF outcome was not affected by whether the retrieved testicular sperm is fresh or frozen (25). Nevertheless, cumulative pregnancy or cumulative live birth was not mentioned in any of the above studies, especially the cumulative live birth. After comparing the outcomes of ICSI-IVF cycles between fresh and frozen-thawed spermatozoa, we found that there were no significant differences in all laboratory parameters, not only fertilization rate and day 3 oocytes utilization rate we mentioned above, but also cleavage rate, rate of the high-score embryo, and the ratio of patients who had no available embryos. The main results we found are consistent with previous studies (12, 21). Then, we conducted a further analysis of clinical outcomes after embryo transfer, including the first embryo transfer cycle and cumulative embryo transfer cycles. Similarly, CPR, LBR, newborns` parameters showed no significant differences between the fresh and frozen groups. Therefore, it could be concluded that using frozen testicular sperm is as effective and safe as fresh testicular sperm in NOA patients with micro-TESE.

4.3 Relatively Poor ICSI Outcomes Were Observed in NOA Patients With Y Chromosome AZFc Microdeletions

Few studies provided a detailed analysis of ICSI-IVF outcomes according to the different pathological types and etiologies of NOA patients (12, 26). Madureira demonstrated no significant differences in ICSI-IVF outcomes between different histopathological subsets in NOA (24). However, in our study, there were significant differences in day 3 utilization rate of MII eggs for ICSI-IVF cycle among NOA patients with different

etiologies (idiopathic= 31.7%, orchitis= 30.8%, cryptorchidism = 27.2%, KS= 24.4%, and lowest in Y chromosome AZFc microdeletions= 22.3%, $p<0.05$). Liu reported that fertilization competent, viable embryo rate, and pregnancy rate of spermatozoa retrieved from men with Y chromosome AZFc chromosome deletions were similar to men without it (27). Our results showed that lower day 3 oocytes utilization rate and high-score embryo rate and lower cumulative CPR and cumulative LBR were observed in patients with Y chromosome AZFc microdeletion, which are similar to Zhang`s report but with less heterogeneity, because the objects we included were all testicular sperm by micro-TESE (28). These results are also consistent with the finding reported by Van and the primary function of the AZFc region in the Y chromosome is involvement in spermatozoa quality or function than in spermatogenesis (29).

4.4 Relatively Poor ICSI Outcomes Were Observed in NOA Patients With Less Sperm Found

Compared to whom got more sperm (>20 approximately), NOA patients with fewer sperm (≤20 approximately) were detected with significantly higher serum FSH level, lower oocytes utilization rate, lower high-score embryo rate, and a higher ratio of cycles without available embryos. Some studies reported that sperm from NOA patients have aneuploidy, mosaicism, and DNA damage that contribute to decreased clinical outcomes (30). Similarly, in this study, a significantly lower clinical pregnancy rate and live birth rate were observed as expected in patients with fewer sperm. These results provide us with a better understanding of treatment outcomes for patients with different laboratory findings after testicular tissue processing.

4.5 Limitations

However, our study still has limitations, including the sample size of the fresh sperm group was not large enough, the need to supplement more follow-up data on live births, and patient selection bias. In the future, multicenter data and randomized controlled trials are needed to determine clinical predictors of successful outcomes for NOA couples.

In conclusion, for those NOA patients who stepped in ICSI-IVF cycles, the cumulative LBR was 46.82%. No significant difference was observed in LBR between ICSI-IVF cycles using frozen or fresh testicular sperm. NOA patients with AZFc microdeletions had the lowest rate of the high-score embryo on day 3 and the lowest cumulative CPR. NOA patients with lower sperm count had significantly lower cumulative LBR than those with higher sperm count.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (reference number 2017-055). Written informed consent was not required for this study, in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

The contributions of all authors were as follow: Conceptualization, Data curation, Writing-original draft: GA. Statistical analysis: HZ. Data collection: YL, XF, TP, CL, JL, and ML. All authors have contributed to critical discussion, reviewed the final version of the manuscript and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.893679/full#supplementary-material

Supplementary Figure 1 | Available embryos of ICSI-IVF in NOA patients with different etiologies.

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Cisplatin Effects on the Human Fetal Testis – Establishing the Sensitive Period for (Pre)Spermatogonial Loss and Relevance for Fertility Preservation in Pre-Pubertal Boys

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Background: Exposure to chemotherapy during childhood can impair future fertility. Studies using *in vitro* culture have shown exposure to platinum-based alkylating-like chemotherapy reduces the germ cell number in the human fetal testicular tissues. We aimed to determine whether effects of exposure to cisplatin on the germ cell sub-populations are dependent on the gestational age of the fetus and what impact this might have on the utility of using human fetal testis cultures to model chemotherapy exposure in childhood testis.

Methods: We utilised an *in vitro* culture system to culture pieces of human fetal testicular tissues (total n=23 fetuses) from three different gestational age groups (14-16 (early), 17-19 (mid) and 20-22 (late) gestational weeks; GW) of the second trimester. Tissues were exposed to cisplatin or vehicle control for 24 hours, analysing the tissues 72 and 240 hours post-exposure. Number of germ cells and their sub-populations, including gonocytes and (pre) spermatogonia, were quantified.

Results: Total germ cell number and number of both germ cell sub-populations were unchanged at 72 hours post-exposure to cisplatin in the testicular tissues from fetuses of the early (14-16 GW) and late (20-22 GW) second trimester. In the testicular tissues from fetuses of mid (17-19 GW) second trimester, total germ cell and gonocyte number were significantly reduced, whilst (pre)spermatogonial number was unchanged. At 240 hours post-exposure, the total number of germ cells and that of both sub-populations was significantly reduced in the testicular tissues from fetuses of mid- and late-second trimester, whilst germ cells in early-second trimester tissues were unchanged at this time-point.

Conclusions: *In vitro* culture of human fetal testicular tissues can be a useful model system to investigate the effects of chemotherapy-exposure on germ cell sub-populations during pre-puberty. Interpretation of the results of such studies in terms of relevance to later (infant and pre-pubertal) developmental stages should take into account the changes in germ cell composition and periods of germ cell sensitivity in the human fetal testis.

Keywords: human, fetal, testis, cisplatin, germ cell, fertility preservation

INTRODUCTION

Chemotherapy treatment for cancer in boys is associated with impaired testicular function and impaired fertility (1). Human-relevant experimental models are important to determine the impacts of specific agents and the influence of developmental stage of the patient (2). We have utilised an *in vitro* system that has been previously shown to maintain testicular tissue integrity when exposing human fetal testicular tissue fragments in hanging drops, to clinically relevant concentrations of pharmaceutical compounds in order to determine the effect of exposure on somatic and germ cell populations (3–5). Using this system, we have previously shown that exposure to cisplatin, a platinum-based chemotherapeutic drug used in the treatment of several paediatric malignancies (6), results in a reduction in total germ cell number in cisplatin-exposed tissues (4).

Whilst spermatogenesis does not occur until adulthood, the germ cell number and composition within the testis undergo changes from fetal life through to puberty (7, 8). Therefore, effects of chemotherapy exposure prior to puberty will be dependent on the germ cell populations present and any changes in sensitivity to treatment. In the human fetal testis the germ cell complement consists of gonocytes and (pre) spermatogonia. Gonocytes predominate during the first trimester and differentiation from gonocyte to (pre) spermatogonia occurs during the second and third trimester (7, 9). During infancy the remaining gonocytes complete differentiation to spermatogonia and for the remainder of childhood, spermatogonia make up the germ cell complement of the testis (8). Therefore, effects of chemotherapy on gonocytes has relevance to the fetal and infantile testis, whilst effects on (pre)spermatogonia are relevant to the testis from late first trimester until adulthood.

A critical period of testicular development during fetal life, known as the masculinisation programming window (MPW), has been identified in rats, during which perturbation of androgens predisposes to the development of male reproductive disorders (10). This resembles the Testicular Dysgenesis Syndrome (TDS) described in human and a similar period of sensitivity to androgens has been proposed to occur between 8-13 weeks gestation (11) TDS includes disorders of germ cell development such as testicular cancer and infertility (12). Whether there are similar critical periods of germ cell sensitivity to chemotherapy in fetal life and if there are differential effects on the germ cell sub-populations during human fetal testis development, has not been reported.

We aimed to determine whether there are specific periods of sensitivity to germ cell loss in the human fetal testis. If this is the case, it might affect how results of *in utero* chemotherapy exposure studies are interpreted. In addition, since the (pre) spermatogonia are the germ cell population present in the pre-pubertal testis, we aimed to determine whether there was a particular gestational age, where the reduction in (pre) spermatogonial numbers was observed.

METHODS

Study Approval

Human fetal tissues were collected after women gave informed consent to donate tissue for research following elective termination of pregnancy (TOP). The work was conducted following ethical approvals (Edinburgh: South East Scotland Research Ethics Committee (LREC08/S1101/1), Newcastle: NRES committee North East – Newcastle and North Tyneside 1 (08/H0906/21+5) and London: NRES Committee London – Fulham (18/10/0822)).

Tissue Collection

Human fetal testicular tissues from 2nd trimester fetuses (total n=23; 14-22 GW) were obtained from elective TOP. Tissues were obtained from Royal Infirmary of Edinburgh and Human Developmental Biology Resource (HDBR) facilities in Newcastle and London. Samples from TOP with known fetal abnormalities were excluded. Gestational age of the fetus was determined by a combination of ultrasound and direct measurement of foot length. The sex of the fetus was confirmed by presence of sex-determining region Y (SRY) gene based on melting curves in qPCR runs using a small piece of skin or limb from each fetus. The number of samples that came from each site of TOP were as follows: London – 17, Newcastle – 4 and Edinburgh – 2. All samples were placed in media (Liebowitz L-15 with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids) and transported to Edinburgh in insulated boxes containing cool-packs. All samples were received within 72 hours of collection. All tissue culture experiments were performed in the same laboratory in Edinburgh.

In Vitro Culture System

Tissue pieces (~1 mm^3) were cultured in hanging drops containing 30 µl droplets of pre-warmed culture media on the upturned lid of

Petri dish as previously described (5). Culture dishes were incubated at 5% CO_2 at 37°C. Culture medium consisted of: Minimum Essential Medium α (MEMα; Lonza), 1x MEM non-essential amino acids (Thermo Fisher Scientific), 2 mM sodium pyruvate (Thermo Fisher Scientific), 2 mM L-glutamine (Life Technologies), 1x Insulin-Transferrin-Selenium (ITS; Sigma-Aldrich), 1x penicillin/streptavidin (Thermo Fisher Scientific) and supplemented with 10% fetal bovine serum (FBS; Life Technologies). The culture media was replaced every 24 hours. For treatments, media was supplemented with cisplatin (0.5 μg/ml) or vehicle (water) for 24 hours beginning on day 3 for 24 hours with analysis at 72 hours or 240 hours after cisplatin-exposure. To generate sufficient samples at each gestational age, we included data from experiments previously reported (4), with additional samples at each gestational age group.

Tissue Processing

Cultured tissue pieces were fixed in Bouin's fluid (Clin-Tech) for 1 hour prior to transfer to 70% ethanol. Fixed samples were embedded in paraffin, sectioned (5μm thickness) and morphology was assessed using H&E according to a standard protocol. All samples included in the analysis showed grossly normal morphology (healthy architecture of tubules, minimal apoptosis and presence of germ cells) in pre-culture and/or vehicle controls. For each treatment a minimum of two sections from two replicate tissue pieces were analysed.

Immunostaining and Cell Quantification

Double colourimetric immunohistochemistry was performed according to previously published protocols (4). Co-staining was performed for expression of AP2γ (gonocytes) and MAGE-A4 ((pre)spermatogonia). Positive controls consisted of pre-culture tissue and negative controls and involved omission of the primary antibody. Positively stained germ cells were manually counted and reported per seminiferous area (mm^2). The assessor remained blinded to treatment group during analysis. Details of antibodies and dilutions are provided in **Table 1**.

Statistics

For each treatment regimen, tissue from each fetus was considered an individual experiment. Fragments from each fetus were cultured and randomly allocated to receive vehicle or cisplatin. No outliers were excluded. Statistical analysis was conducted using GraphPad Prism 8 software (GraphPad Software Inc., USA). Two-way analysis of variance (ANOVA) with multiple comparisons using Bonferroni's *post hoc* test was performed, accounting for treatment group (vehicle or cisplatin) and individual sample (fetus) as two independent variables (13). Data are presented as mean ± standard error of mean (SEM) for all fragments from each fetus and each distinct colour-coded point or square represents an individual fetus. Statistical significance was defined as $p<0.05$.

RESULTS

Exposure to Cisplatin- Induced Germ Cell Loss in Human Fetal Testis Tissues

Normal testicular morphology was maintained in control (**Figures 1A, C**) and cisplatin-exposed (**Figures 1B, D**) human fetal testicular tissues, with preservation of seminiferous cords and presence of germ cells at 72 (**Figures 1A, B**) and 240 (**Figures 1C, D**) hours post-exposure. Immunohistochemistry was carried out for AP2γ (gonoytes) and MAGE-A4 ((pre) spermatogonia) cells (**Figures 2A, B, F, G**) and used for quantification. At 72 hours, cisplatin exposure resulted in a significant reduction in the total number of germ cells (639 ± 82 vs 763 ± 83 cells/cord area (mm^2), $p<0.01$; **Figure 2C**) and gonocytes (296 ± 70 vs 385 ± 74 cells/cord area (mm^2), $p<0.001$; **Figure 2D**), whilst (pre)spermatogonial cell number (**Figure 2E**) was unchanged, compared to controls. At 240 hours, cisplatin exposure resulted in a significant reduction in total germ cell (516 ± 64 vs 688 ± 45 cells/cord area (mm^2), $p<0.0001$; **Figure 2H**), gonocyte (163 ± 30 vs 281 ± 36 cells/cord area (mm^2), $p<0.0001$; **Figure 2I**), and (pre)spermatogonial cell (357 ± 50 vs 408 ± 33 cells/cord area (mm^2), $p<0.05$; **Figure 2J**) number, compared to controls.

Cisplatin-Induced Germ Cell Loss is Dependent on the Gestational Age of the Fetus

The (pre)spermatogonial number is unchanged at 72 hours and only marginally reduced at 240 hours. Given the importance of this population when extrapolating results from the fetal testis to human pre-pubertal testis, we analysed according to three gestational age groups (early-, mid- and late-second trimester. At 72 hours post-exposure to cisplatin, there was no difference in total germ cell number or the germ cell sub-populations from fetuses obtained during early (14-16 GW; **Figures 3A, D, G**) or late (20-22 GW; **Figures 3C, F, I**) second trimester testicular tissue. However, there was a reduction in total germ cell number

TABLE 1 | Summary of immunohistochemistry protocol.

Antibody (Cat no)	Method	Dilution (antigen retrieval)	Origin	Blocking agent	Detection
AP2γ (sc-12762)	IHC	1:20 (citrate buffer)	Mouse	Normal horse serum/ TBS/BSA	DAB
MAGE-A4 (Gift from Giulio Spagnoli)	IHC	1:40 (citrate buffer)	Mouse	Normal horse serum/ TBS/BSA	Vector Blue

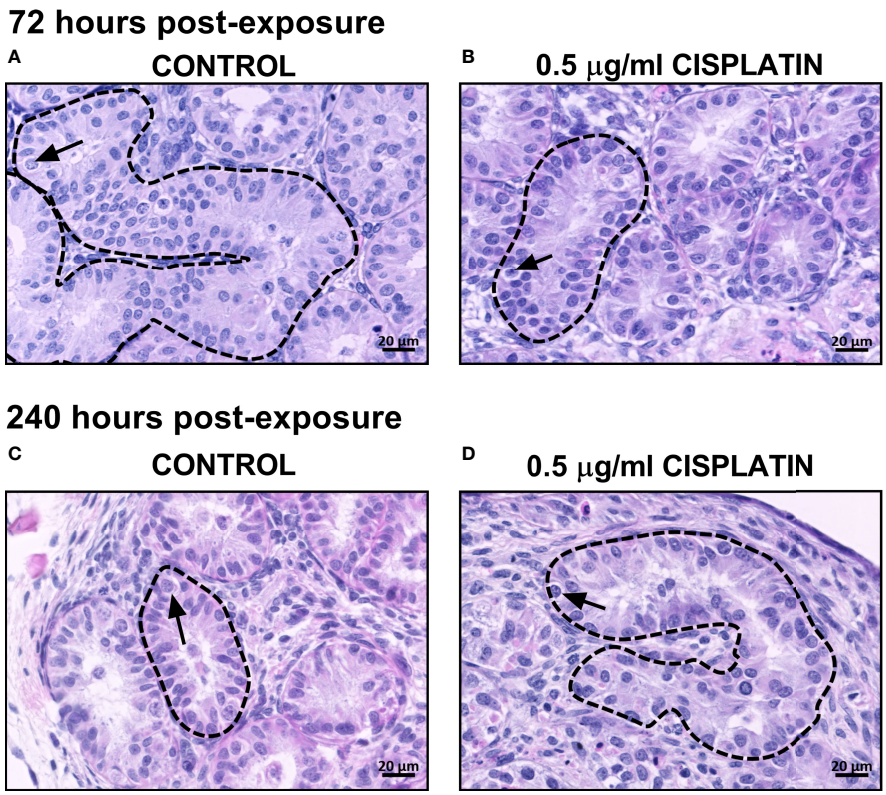

FIGURE 1 | Effects of exposure to cisplatin on the gross morphology of human fetal testicular tissues at 72 and 240 hours post-exposure. H&E staining in vehicle control **(A, C)** and cisplatin-exposed **(B, D)** tissues at 72 and 240 hours post-exposure. Dotted lines outline seminiferous cords and arrows point to germ cells. Scale bars represent 20 μm.

(491 ± 80 vs 646 ± 106 cells/cord area (mm^2), $p<0.05$; **Figure 3B**) in mid-second trimester samples in the cisplatin-exposed tissues, compared to control. A differential effect on the sub-populations was demonstrated at this gestation with a significant reduction in gonocytes (173 ± 31 vs 307 ± 94 cells/cord area (mm^2), $p<0.0001$; **Figure 3E**), whilst the number of (pre)spermatogonial (**Figure 3H**) was unchanged in cisplatin-exposed tissues, compared with control.

At 240 hours post-exposure to cisplatin, there was no difference in total germ cell number from fetuses obtained during early (14-16 GW; **Figure 4A**) second trimester. There was a significant reduction in gonocytes (266 ± 68vs 430 ± 73 cells/cord area (mm^2), $p<0.0001$; **Figure 4D**) after exposure to cisplatin at this stage, whilst the (pre)spermatogonia were unaffected (**Figure 4G**). For mid-second trimester tissues, there was a significant reduction in total germ cell number (395 ± 71 vs 627 ± 73 cells/cord area (mm^2), $p<0.0001$; **Figure 4B**), gonocytes (101.2 ± 39.5 vs 238 ± 48 cells/cord area (mm^2), $p<0.01$; **Figure 4E**) and (pre)spermatogonia (303 ± 63 vs 393± 64 cells/cord area (mm^2), $p<0.05$; **Figure 4H**) in cisplatin-exposed tissues, compared with control (**Figure 4B**). Similarly, for late-second trimester tissues, cisplatin exposure resulted in a significant decrease in total germ cells (458 ± 98 vs 616 ± 73 cells/cord area (mm^2), $p<0.001$, **Figure 4C**), gonocytes (151 ± 40 vs 218 ± 45 cells/cord area (mm^2), $p<0.01$; **Figure 4F**) and (pre) spermatogonia (308 ± 66 vs 398 ± 44 cells/cord area (mm^2), $p<0.01$; **Figure 4I**).

DISCUSSION

The aim of this study was to determine whether cisplatin effects on the germ cell sub-populations was dependent on the gestational age of fetus from which the testicular tissues were obtained. The results in this study indicate the effects of cisplatin exposure on germ cell number in second trimester human fetal testis are restricted to the middle and late time-points. This suggests a specific period of susceptibility to germ cell loss following cisplatin exposure. These data may have implications for understanding the effects of chemotherapy treatment in pregnant women on the fetus. In addition, these findings are important when interpreting the results from studies that include tissues from fetuses across the whole gestational period and, moreover, are essential for the design of future studies aimed at using human fetal testis cultures to model the effects of chemotherapy on spermatogonia in the pre-pubertal testis.

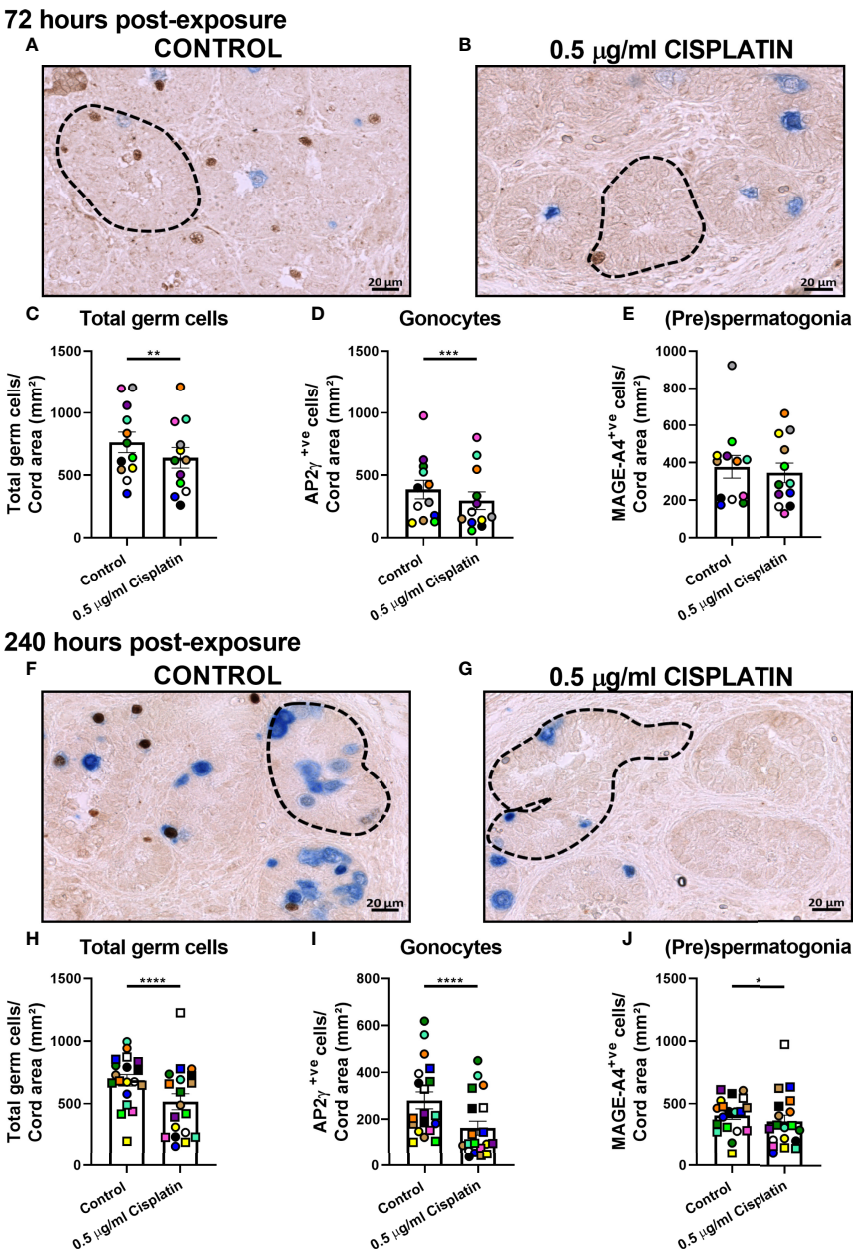

FIGURE 2 | Pooled data on effects of exposure to cisplatin on germ cell number in human fetal testicular tissues at 72 and 240 hours post-exposure. Immunohistochemical staining for gonocytes (AP2γ+; brown) and (pre)spermatogonia (MAGEA4+; blue) in the human fetal testis in vehicle control **(A)** and cisplatin-exposed **(B)** tissues at 72 hours; and in vehicle control **(F)** and cisplatin-exposed **(G)** tissues at 240 hours post-exposure. Scale bars represent 20 μm. Dotted lines outline seminiferous cords. Total number of germ cells **(C)**, gonocytes **(D)** and (pre)spermatogonial **(E)** at 72 hours post-exposure. Total number of germ cells **(H)**, gonocytes **(I)** and (pre)spermatogonial **(J)** at 240 hours post-exposure. Data analysed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Values shown are means ± SEM and each data point represents the mean value for all fragments obtained from an individual fetus (n = 12-19).

Impacts of *In Utero* Exposure to Cisplatin on Human Fetal Testis – Windows of Sensitivity of Germ Cell Sub-Populations

Chemotherapy administration during pregnancy may result in spontaneous abortion and fetal death. The potential for fetal death is highest in the first trimester and reduces as pregnancy progresses (14). In a large (n=1170 women) cohort study of obstetric and neonatal outcomes in women diagnosed with cancer during pregnancy, 88% of the pregnancies resulted in a livebirth (15). A recent review reported livebirth in all 36 pregnant women treated with platinum-based chemotherapy (16). However, despite the relatively high survival of chemotherapy-exposed fetuses, there are no reports on the impacts of intrauterine cisplatin exposure on gonadal

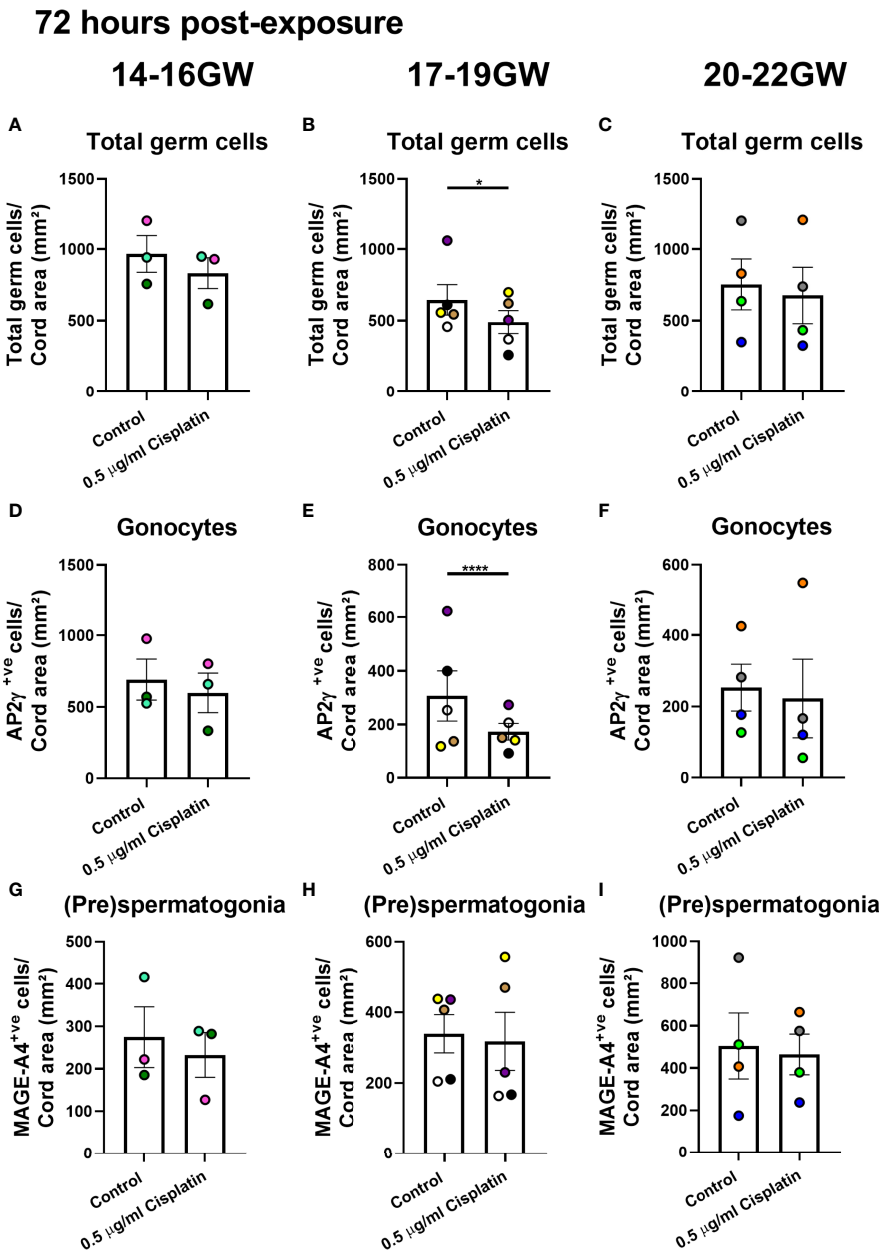

FIGURE 3 | Effects of exposure to cisplatin on germ cell number in human fetal testicular tissues at 72 hours post-exposure according to gestational age. Total number of germ cells in early- **(A)**, mid- **(B)** and late- **(C)** second trimester human fetal testis. Gonocyte number in early- **(D)**, mid- **(E)** and late- **(F)** second trimester human fetal testis. (Pre)spermatogonial number in early- **(G)**, mid- **(H)** and late- **(I)** second trimester human fetal testis. Data analysed using two-way ANOVA. *$p < 0.05$, ****$p < 0.0001$. Values shown are means ± SEM and each data point represents the mean value for all fragments obtained from an individual fetus (n = 12).

development and function. Our previous study involving xenografting of cisplatin-exposed human fetal testis demonstrated that germ cell loss persists over time and the degree of germ cell loss (~50% reduction in cisplatin-exposed compared with control), is similar at 3 months in xenografts, to that observed after short-term culture (4). The present results indicate that gonocytes are susceptible to loss following cisplatin exposure during the majority of the second trimester. The persistent sensitivity of this cell population may impact on the ability of gonocytes to contribute to the (pre)spermatogonial pool. The present study also suggests that the most sensitive period for the (pre)spermatogonial population appears to occur during the middle to late stages of the second trimester. The difference in sensitivity between the germ cell populations may

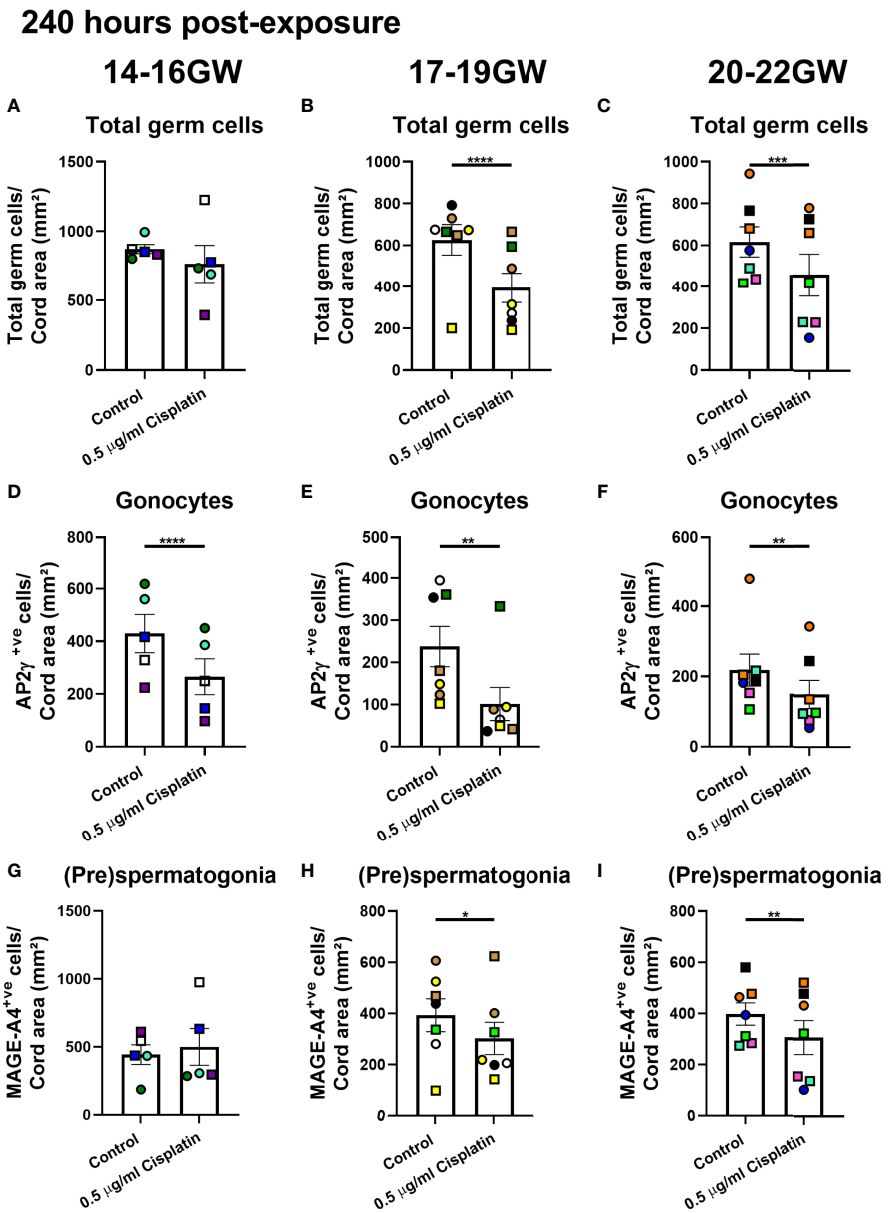

FIGURE 4 | Effects of exposure to cisplatin on germ cell number in human fetal testicular tissues from different gestational age groups at 240 hours post-exposure according to gestational age. Total number of germ cells in early- **(A)**, mid- **(B)** and late- **(C)** second trimester human fetal testis. Gonocyte number in early- **(D)**, mid- **(E)** and late- **(F)** second trimester human fetal testis. (pre)spermatogonial number in early- **(G)**, mid- **(H)** and late- **(I)** second trimester human fetal testis. Data analysed using two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Values shown are means ± SEM and each data point represents the mean value for all fragments obtained from an individual fetus (n = 19).

relate to variation in the proliferation rate, 20-30% in gonocytes compared with 0-5% in (pre)spermatogonia, between these two populations during the second trimester (17).

Impacts of Chemotherapy Exposure of Human Fetal Testis as a Proxy for the Childhood Testis

Germ cell differentiation from gonocyte to spermatogonia occurs asynchronously during fetal and early postnatal life (7, 18). During the first trimester, the majority of germ cells are defined as gonocytes based on the expression of markers including POU5F1 (pluripotency factor) and AP2γ. During the second trimester however, the proportion of gonocytes reduces as the (pre) spermatogonial (MAGE-A4) population increases (7). In infancy, the remaining gonocytes differentiate into spermatogonia and subsequently the germ cell complement consists of spermatogonia, until the onset of spermatogenesis at puberty (8, 18). Therefore, extrapolation of the results of studies involving the *in*

vitro human fetal testis model to the infant or childhood testis must take account of the germ cell composition and periods of sensitivity for the germ cell sub-populations. The present results demonstrate the importance of gestational age in designing studies to determine effects of chemotherapy-exposure or protection from chemotherapy-induced germ cell loss. Studies aimed at understanding the effects of chemotherapy exposure on the human infant and pre-pubertal testis, should focus on a relevant developmental age with respect to the (pre)spermatogonial population. The present results suggest that the optimal time-point for these human fetal testis tissues is from the mid-second trimester onwards.

CONCLUSIONS

In vitro culture of human fetal testis can be a useful model system to investigate the effects of chemotherapy-exposure on germ cell sub-populations during pre-puberty. Interpretation of the results of such studies, in terms of relevance to later (infant and pre-pubertal) developmental stages, should take into account the changes in germ cell composition and periods of germ cell sensitivity in the human fetal testis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Edinburgh: South East Scotland Research Ethics Committee (LREC08/S1101/1), Newcastle: NRES committee North East – Newcastle and North Tyneside 1 (08/H0906/21 +5) and London: NRES Committee London – Fulham (18/10/0822). The patients/participants provided their written informed consentto participate in this study.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GM, RM. Performed the experiments: GM. Analysed the data: GM, RTM. Feedback on the manuscript: MR, NS, RA. Wrote the paper: GM, RTM. All authors approved the final submission. RA and RM jointly supervised PhD student GM.

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