Human Reproduction, Vol.34, No.12, pp. 2443–2455, 2019
Advance Access Publication on December 20, 2019 doi:10.1093/humrep/dez180

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Assessment of fresh and cryopreserved testicular tissues from (pre)pubertal boys during organ culture as a strategy for *in vitro* spermatogenesis

J.M.D. Portela, C.M. de Winter-Korver, S.K.M. van Daalen, A. Meißner, A.A. de Melker, S. Repping, and A.M.M. van Pelt*

Center for Reproductive Medicine, Research Institute Reproduction and Development, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

*Correspondence address. Center for Reproductive Medicine, Research Institute Reproduction and Development, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail: a.m.vanpelt@amsterdamumc.nl

Submitted on January 11, 2019; resubmitted on July 26, 2019; editorial decision on July 31, 2019

STUDY QUESTION: Can the organ culture method be applied to both fresh and cryopreserved human (pre)pubertal testicular tissue as a strategy for *in vitro* spermatogenesis?

SUMMARY ANSWER: Although induction of spermatogenesis was not achieved *in vitro*, testicular architecture, endocrine function and spermatogonial proliferation were maintained in both fresh and cryopreserved testicular tissues.

WHAT IS KNOWN ALREADY: Cryopreservation of a testicular biopsy is increasingly offered as a fertility preservation strategy for prepubertal cancer patients. One of the proposed experimental approaches to restore fertility is the organ culture method, which, in the mouse model, successfully allows for *in vitro* development of spermatozoa from testicular biopsies. However, complete spermatogenesis from human prepubertal testicular tissue in such an organ culture system has not been demonstrated.

STUDY DESIGN, SIZE, DURATION: Testicular tissue was collected from nine (pre)pubertal boys diagnosed with cancer (ranging from 6 to 14 years of age) admitted for fertility preservation before treatment. Testicular biopsies were either immediately processed for culture or first cryopreserved, using a controlled slow freezing protocol, and thawed before culture. Organ culture of testicular fragments was performed in two different media for a maximum period of 5 weeks, targeting early cellular events (viability, meiosis and somatic differentiation) *in vitro*.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Fresh and cryopreserved-thawed testis fragments (1–2 mm^3) were cultured at a gas–liquid interphase (34°C, 5% CO_2) in Minimum Essential Medium alpha + 10% knock-out serum replacement medium containing 10^{-7} M melatonin and 10^{-6} M retinoic acid, with or without 3 IU/L FSH/LH supplementation. The effect of culture conditions on testicular fragments was weekly assessed by histological evaluation of germ cell development and immunohistochemical identification of spermatogonia (using MAGEA4), proliferative status of spermatogonia and Sertoli cells (using proliferating cell nuclear antigen [PCNA]), intratubular cell apoptosis (by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) and Sertoli cells maturation (using Anti-Müllerian Hormone [AMH] versus Androgen Receptor [AR]). Additionally, Leydig cells' functionality was determined by measuring testosterone concentration in the culture media supernatants.

MAIN RESULTS AND THE ROLE OF CHANCE: Neither fresh nor cryopreserved human (pre)pubertal testicular fragments were able to initiate spermatogenesis in our organ culture system. Nonetheless, our data suggest that fresh and cryopreserved testicular fragments have comparable functionality in the described organ culture conditions, as reflected by the absence of significant differences in any of the weekly evaluated functional parameters. Additionally, no significant differences were found between the two tested media when culturing fresh and cryopreserved human testicular fragments. Although spermatogonia survived and remained proliferative in all culture conditions, a significant reduction of the spermatogonial population ($P \leq 0.001$) was observed over the culture period, justified by a combined reduction of proliferation activity ($P \leq 0.001$) and increased intratubular cell apoptosis ($P \leq 0.001$). We observed a transient increase in Sertoli cell proliferative activity, loss of AMH expression ($P \leq 0.001$) but no induction of AR expression. Leydig cell endocrine function was successfully stimulated *in vitro* as indicated by increased testosterone production in all conditions throughout the entire culture period ($P \leq 0.02$).

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Although not noticeable in this study, we cannot exclude that if an optimized culture method ensuring complete spermatogenesis in human testicular fragments is established, differences in functional or spermatogenic efficiency between fresh and cryopreserved tissue might be found.

WIDER IMPLICATIONS OF THE FINDINGS: The current inability to initiate spermatogenesis *in vitro* from cryopreserved human testicular fragments should be included in the counselling of patients who are offered testicular tissue cryopreservation to preserve fertility.

STUDY FUNDING/COMPETING INTEREST(S): This project was funded by EU-FP7-PEOPLE-2013-ITN 603568 'Growsperm'. None of the authors have competing interests.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: (pre)pubertal human testicular tissue / cryopreservation / *in vitro* / organ culture / fertility restoration

Introduction

Treatment success of paediatric cancers has considerably increased over recent years, but as a consequence, male childhood cancer survivors often experience long-term comorbidities including infertility (Wallace, 2011; Phillips *et al.*, 2015). Oncological treatments involving chemotherapeutic agents and radiation can severely impair the gonads by direct damage to the spermatogonial population, which represents the basis for spermatogenesis (Schrader *et al.*, 2001; Jahnukainen *et al.*, 2011; Wallace, 2011; Anderson *et al.*, 2015). The extent of damage and consequent risk of infertility later in life substantially depends on treatment type and duration and received dosage (Schrader *et al.*, 2001; Meistrich, 2013; Stukenborg *et al.*, 2018). Prepubertal boys at risk of fertility impairment should be considered for fertility preservation strategies prior to receiving gonadotoxic treatment. Because of the absence of mature gametes, the current option to preserve fertility potential of these patients relies on cryopreservation of a testicular biopsy harbouring unharmed spermatogonial stem cells (SSCs) (Anderson *et al.*, 2015; Picton *et al.*, 2015).

Cryopreservation of testicular tissue is increasingly offered in various specialized centres worldwide, whereas fertility restoration strategies to derive sperm from cryopreserved immature human testicular biopsies remain under development (Picton *et al.*, 2015). In animal models including rodents and non-human primates, a number of potential techniques are being investigated and production of sperm and/or offspring has already been proven following autologous SSC transplantation (Brinster and Avarbock, 1994; Hermann *et al.*, 2012), testicular tissue grafting (Honaramooz *et al.*, 2002, 2004; Liu *et al.*, 2016; Shetty *et al.*, 2018; Ntemou *et al.*, 2019; Fayomi *et al.*, 2019) and *in vitro* spermatogenesis by organ culture (Sato *et al.*, 2011a, 2011b).

In 2011, Sato and colleagues reported for the first time successful *in vitro* differentiation of fresh murine neonatal spermatogonia into functional sperm by employing an organ culture method (Sato *et al.*, 2011a, 2011b). This approach, consisting of culturing small testicular fragments positioned on a supportive stand at the gas–liquid interface, was later applied to cryopreserved neonatal mice testicular tissue (Yokonishi *et al.*, 2014; Dumont *et al.*, 2015) and adult mice testicular tissue (Sato *et al.*, 2015). Despite the encouraging results, generation of sperm *in vitro* from human prepubertal testicular tissue has not yet been accomplished (de Michele *et al.*, 2018b; Medrano *et al.*, 2018). Furthermore, to become clinically applicable, the organ culture method has to be successfully applied to cryopreserved testicular material as tissue cryopreservation is a routine element in fertility preservation strategies.

This study aimed to determine whether the organ culture method can be applied to fresh and cryopreserved human (pre)pubertal testicular tissue as a strategy for *in vitro* spermatogenesis. To examine if the low success of human (pre)pubertal testicular organ culture (de Michele *et al.*, 2018b; Medrano *et al.*, 2018) was due to cryopreservation-induced damaged to the tissue, we compared functionality of fresh and cryopreserved-thawed tissue in culture medium without or supplemented with FSH and LH.

Materials and Methods

Ethical approval

Testicular tissue was collected from 9 (pre)pubertal boys (6–14 years, Table I) admitted at the Academic Medical Center (Amsterdam, The Netherlands) for fertility preservation procedure prior to gonadotoxic cancer treatment. For all cases, ethical approval (NL27690.000.09) was obtained from the National Ethical Committee (CCMO), and informed consent was received from patients' parents for using a portion of the testicular biopsy for research purposes.

Testicular tissue collection and cryopreservation

Patients underwent open testicular biopsy during deep venous catheter insertion with general anaesthesia. The main part of each biopsied testicular tissue was cryopreserved and stored for potential future clinical application and a portion of ~15% was used for research. Biopsied testicular tissue for research was either immediately processed or first cryopreserved before organ culture (Fig. 1). Cryopreservation of testicular fragments was performed following a slow freezing protocol as previously described (Keros *et al.*, 2007). Briefly, testicular biopsies were cut into small fragments and preserved in straws with 5% dimethyl sulfoxide (Cryosure—DMSO; WAK Chemie Medical GmbH, Germany) and 5% albumin (Albuman; Sanquin, The Netherlands) in human tubal fluid-HEPES (Gynotec bv., The Netherlands). Samples were stored in vapour phase nitrogen and used for research between 0.5 and 2 months after freezing.

Organ culture of testicular tissue

Cryopreserved tissue samples were thawed and immediately washed three times in Minimum Essential Medium alpha (α-MEM; 22561-021,

Table I Baseline and histological characteristics of testicular tissues collected from nine (pre) pubertal patients.

Organ culture code	Age (years)	Diagnosis	Testicular histology before culture			
			Most advanced germ cell	Average Johnsen score #	AMH expression	AR expression
OCID22 *	6	Rhabdomyosarcoma	Spermatogonia	2.6	Yes	No
OCID30	8.5	Intracranial, spinal embryonal tumor	Spermatogonia	2.7	Yes	No
OCID25	9	Lymphocytic leukemia	Spermatogonia	2.4	Yes	No
OCID23	10	Rhabdomyosarcoma	Spermatogonia	2.3	Yes	Dim
OCID24	10	Glioma	Spermatogonia	2.9	Yes	No
OCID27	10.5	Anaplastic Large Cell Lymphoma	Spermatogonia	2.9	Yes	No
OCID29 *	11	Rhabdomyosarcoma	Few spermatogonia (<25%)	2.2	Yes	No
OCID26 *	12	Intracranial, spinal embryonal tumor	Few spermatogonia (<25%)	2.2	Yes	No
OCID28	14	M. Hodgkin	Elongating spermatids	6.0	No	Moderate

*Testicular histological characteristics evaluated in cryopreserved-thawed tissue.
#Calculation of average Johnsen score (Johnsen, 1970) is described in Supplementary Table SI.

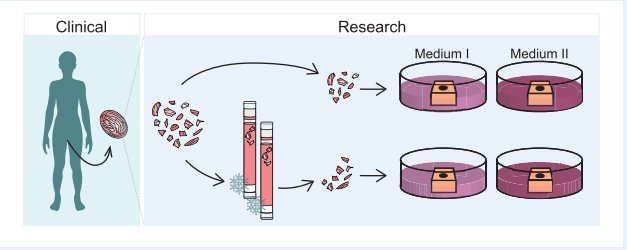

Figure 1 Schematic overview of the experimental design. Biopsied testicular tissue for research was immediately processed or first cryopreserved in straws before organ culture. Testis fragments (1–2 mm^3) were cultured on top of agarose stands for a maximum of 5 weeks (34°C, 5% CO_2) in two different media without (medium I) or with hormonal supplementation (medium II).

Thermo Fisher Scientific, USA) on ice before being further processed for culture.

Fresh or cryopreserved-thawed testis fragments were processed into 1–2 mm^3 pieces. Prior to initiation of culture, one fragment was fixed in modified methacarn (89% methanol and 11% glacial acetic acid) for 3 hours at room temperature (RT) (Jan *et al.*, 2017). The remaining fragments were divided into two groups for culture in two media: culture medium I was composed of α-MEM + 10% knockout serum replacement (KSR; 10828-028, Thermo Fisher Scientific, USA) + 1% penicillin-streptomycin (15140122, Thermo Fisher Scientific, USA) supplemented with 10^{-7} M melatonin (M5250, Sigma-Aldrich, Germany) and 10^{-6} M retinoic acid (R2625, Sigma-Aldrich, Germany) and culture medium II consisted of culture medium I with the addition of 3 IU/L FSH/LH (Menopure 75 IE, Ferring B.V., The Netherlands) (Fig. 1). Testicular fragments were placed on top of 0.35% (w/v) agarose (seaKem LE; 50004, Lonza, Switzerland) stands according to the gas–liquid interphase method. Agarose stands were prepared by mixing equal volumes of pre-autoclaved 0.7% (w/v) agarose with culture medium. After solidification, agarose stands were cut and transferred into 6-well plates. Each agarose stand was loaded with up to three fragments, and the culture medium was adjusted to the edge of the stand without covering the testicular fragments (Reda *et al.*, 2017). To focus on early events crucial for spermatogenesis initiation and further progress *in vitro* (cell viability, meiosis initiation and somatic differentiation), testicular pieces were cultured for a maximum period of 5 weeks (Table II; Supplementary Table SII) at 34°C and 5% CO_2. Culture media were renewed weekly, and supernatants were collected. Depending on the initial biopsy size, a minimum of three cultured pieces were weekly fixed in modified methacarn and paraffin (ParaPlast Plus—39602004; Leica Biosystems, Germany) embedded.

Immunohistochemistry

To characterize pre-culture tissue and assess the effect of culture conditions on testicular fragments, several immunohistochemical stainings were performed on 5 µm sections. After deparaffinization, antigen retrieval was performed, if necessary, in 0.01 M sodium citrate buffer (pH 6.0) in a microwave for 10 minutes. Endogenous peroxidases were inactivated with 0.3% H_2O_2 and nonspecific adhesion sites blocked with suitable blocking solution (Supplementary Table SIII) for 1 hour at RT. Sections were subsequently incubated overnight at 4°C with mouse monoclonal primary antibodies (Supplementary Table SIII) for anti-MAGEA4 (1:100; kindly provided by Prof. Spagnoli) as a spermatogonial marker, anti-PCNA (1:200; ab92552, Abcam, UK) as a proliferation marker, anti-γ-H2AX (1:20 000; 05-636, Millipore, USA) as meiotic marker, anti-AMH (1:100; sc-166 752, Santa Cruz Biotechnology, USA) as immature Sertoli cell marker or anti-AR (1:100; SC-7305, Santa Cruz Biotechnology, USA) as mature Sertoli cell marker. After washes in PBS, sections were incubated with secondary antibody Poly-HRP-Goat anti Ms/Rb/Rt IgG (DPVO110HRP, Immunologic BV, The Netherlands).

Table II Detailed information on culture period (weeks) and total number of cultured testicular fragments analysed for each (pre)pubertal patient.

Organ culture code	Age (years)	Time points of analysis (weeks)		Total number of fragments (detailed in Supplementary Table SII)
		Fresh	Cryopreserved-thawed	
OCID22	6	2, 4, 5	0, 2, 4, 5	40
OCID30	8.5	0, 1, 2, 4, 5	0, 1, 2, 4, 5	48
OCID25	9	0, 2, 4	0, 2, 4	25
OCID23	10	0 to 5	0, 1, 2, 4, 5	54
OCID24	10	0 to 5	0, 1, 2, 4, 5	56
OCID27	10.5	0, 2, 4, 5	0, 2, 4, 5	35
OCID29	11	2, 4, 5	0, 2, 4, 5	38
OCID26	12	1 to 5	1, 2, 4, 5	56
OCID28	14	0 to 5	0 to 5	62

Depending on initial biopsy size, the number of cultured fragments and distribution throughout culture conditions and time points of analysis were variable between patients. Due to tissue scarcity, solely for one patient (OCID28) were all culture conditions evaluated at week 3.

Signal was visualized with 3,3′-diaminobenzidine (Bright-DAB; BS04, Immunologic BV, The Netherlands).

Proliferating spermatogonia were recognized with anti-MAGEA4 and anti-PCNA double staining following the manufactures protocol (Polink DS-MR-Hu D1 kit, DS201D-18, GBI Labs, USA). MAGEA4 and PCNA signals were visualized with DAB and fast-red chromogens, respectively.

For all stainings, negative controls were performed using isotype IgG in corresponding concentrations. All sections were counterstained with Mayer's haematoxylin.

Stained sections were entirely scanned using Philips IntelliSite UFS slide scanner (Philips Digital Pathology Solutions, The Netherlands) at 40× magnification.

Intratubular cell apoptosis

For histological identification of apoptotic cells, a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay was performed on sections of cultured and uncultured testicular fragments according to the manufacturer's protocol (*In Situ* Cell Death Detection Kit, 11684817910, Roche Diagnostics, The Netherlands), with the enzyme solution diluted 1:100 in PBS. Positive controls were established on adult human testis sections treated with 2 mg/mL DNAse, while sections processed without enzyme solution in the TUNEL reaction mix were used as negative controls. Visualization of apoptotic cells was done using DAB as a chromogen, and sections were counterstained with Mayer's haematoxylin.

Characterization of the cultured testicular tissue fragments

All testicular fragments were evaluated for the presence of the most advanced germ cell and (immuno)histochemical detection of spermatogonia, proliferative status of spermatogonia and Sertoli cells, intratubular cell apoptosis and maturation status of Sertoli cells. Germ and Sertoli cells were identified based on morphological nuclear criteria and location within the seminiferous epithelium (Clermont, 1972).

For each staining, semi-quantitative analysis on up to five fragments per condition and time point (Supplementary Table SII) was blindly assessed on two cross-sections of every fragment (Supplementary Table SIV) with at least 20 µm interval distance assuring evaluation of independent cells. Due to tissue scarcity, enough fresh and cryopreserved material was available to perform experiments at all culture conditions at week 3 for only one patient (OCID28) (Table II; Supplementary Table SII).

All tubular cross-sections were counted, and the results for each marker were expressed as the ratio between the number of tubules with at least one positive cell and the total number of tubules. A total of 370–1145 tubular cross-sections were evaluated per patient for MAGEA4/PCNA double-stained sections, 469–1064 for AMH and 690–1199 for TUNEL (Supplementary Table SIV).

Results for each patient and culture condition were individually represented in R (version 3.4.0) using the *ggplot2* package. The boxplot displays the median and first and third quartiles (25th and 75th percentiles) of all patients at each time point in culture. Where relevant, the cohort was divided into patients with complete spermatogenesis (Johnsen score 6) and those with spermatogonia as the most advanced germ cell type (Johnsen score 2–3).

Measurement of testosterone secretion to culture media

Culture supernatants were weekly collected and stored at −20°C until further use. Testosterone levels in culture supernatants were measured in duplicate using Parameter Testosterone Immunoassay (SKGE010/KGE010, R&D Systems, USA) following the manufacturers protocol. Concentrations (ng/mL) were calculated according to the optical density at 450 and 560 nm (microplate reader Multiskan EX, Thermo Fisher Scientific, Finland) compared to a standard curve. The sensitivity of the assay was 0.03 ng/mL with intra- and inter-assay coefficients of variation of $<5\%$ and $<10\%$, respectively.

Calculated values were normalized by the number of fragments present in the culture wells at time of supernatants collection. The results are presented as the mean ± SE of all patients at each time point in culture.

Statistical analyses

Statistical analyses were performed in SPSS Statistics for Windows, Version 22.0 (IBM Corp., USA). The influence of culture media, initial state of the tissue (fresh versus cryopreserved) and culture period on the functional parameters of testicular fragments (spermatogonial survival and proliferation, intratubular cell apoptosis, Sertoli cell maturation and proliferation) were determined by a mixed-effect linear model, with variables considered as fixed effects. Interaction effect between the variables, culture media, initial state of the tissue (fresh versus cryopreserved) and culture period, was always analysed but removed from the model when not significant ($P > 0.05$).

For evaluation of testosterone concentration in the various culture conditions and throughout culture period, three-way ANOVA for repeated measurements followed by Bonferroni tests were performed.

Differences were considered significant for a *P*-value of <0.05. No outliers were excluded.

Results

Testicular tissue maturation status before culture

Testicular tissues collected from nine patients, who were admitted for fertility preservation procedure before gonadotoxic treatment, showed histological heterogeneity concerning the most advanced germ cell content and Sertoli cells maturation status. A prepubertal status was demonstrated in the majority of boys (ages between 6 and 12 years old), with spermatogonia being the only germ cell type present in the seminiferous tubules, without evidence of initiation of spermatogenesis (Johnsen score 2–3; Supplementary Table SI). There is only one patient of 14 years of age (OCID28), and he showed complete spermatogenesis, with all germ cell types constituting the seminiferous epithelium (Johnsen score 6; Supplementary Table SI). For all patients without spermatogenesis progression, Sertoli cells were still immature, as observed by the expression of AMH. However, patient OCID23 showed simultaneous expression of AMH and dim expression of AR, suggesting an intermediate maturation phenotype. When spermatogenesis was already ongoing (patient OCID28), a mature state of Sertoli cells was confirmed by the absence of expression of AMH and moderate expression of AR (Table I).

Most advanced germ cell type after organ culture

For all prepubertal patients with spermatogonia as the most advanced germ cell type in the biopsied testicular tissue, organ culture failed to induce spermatogonial differentiation, irrespectively of the conditions tested. The results were confirmed morphologically and by the absence of immunohistochemical staining of the meiotic marker γ-H2AX (data not shown). For the pubertal patient (OCID28), γ-H2AX expression was lost after 1 week in organ culture (data not shown). Additionally, spermatogenesis was not maintained *in vitro* and, in fact, depletion of more differentiated layers of the seminiferous epithelium and tubular sclerosis was evident throughout culture while elongating spermatids were maintained for 5 weeks (Supplementary Fig. S1a).

Spermatogonial survival during testicular culture

Based on MAGEA4 immunohistochemical evaluation, spermatogonia survived *in vitro* throughout the culture period of 5 weeks (Fig. 2a). However, quantitative analysis revealed a statistically significant reduction ($P \leq 0.001$) in the percentage of seminiferous tubules containing at least one MAGEA4 positive cell during culture.

Spermatogonial survival was comparable between fresh and cryopreserved cultured fragments and was independent of the culture medium hormonal supplementation (Fig. 2b).

Spermatogonial proliferation during testicular culture

The percentage of tubules containing at least one cell simultaneously expressing MAGEA4 and PCNA (Fig. 3a) was used as the spermatogonial proliferation index. Despite spermatogonia remained proliferative *in vitro*, a significant decrease in the percentage of tubules containing at least one proliferating spermatogonia was observed throughout culture ($P \leq 0.001$). Furthermore, no statistical difference in tubular cross-sections with proliferating spermatogonia was observed between fresh and cryopreserved cultured fragments nor between the two culture media tested (Fig. 3b).

Fragments initially containing elongating spermatids (OCID28) showed less proliferation of spermatogonia in testicular tissue culture, becoming absent after 1 week in medium with FSH/LH (Medium II) and after 2 weeks in medium without hormonal supplementation (Medium I) (Supplementary Fig. S1a and b).

Sertoli cells proliferation during testicular culture

Sertoli cells were morphologically identified and proliferation activity was determined by immunohistochemical expression of PCNA (Fig. 3a). An increase of Sertoli cells proliferative activity was evident after one week of testicular culture, followed by a decrease and stabilization, even though no statistical significance was reached due to the non-linear behaviour of repeated measurements. In fact, for the overall culture period, a significant decrease ($P \leq 0.001$) in the percentage of tubules with at least one proliferating Sertoli cell was observed (Fig. 3c).

Although average Sertoli cell proliferation was higher in testicular fragments that had been cryopreserved before culture compared to freshly cultured fragments, the values were not statistically different for the global culture period. Additionally, no statistical difference was detected between the two culture media tested.

Intratubular cell apoptosis

Intratubular cell apoptosis was considered since morphological distinction between TUNEL-positive spermatogonia or Sertoli cells was hampered by compromised morphological integrity of the apoptotic cell nucleus. A significant increase ($P \leq 0.001$) in the percentage of intratubular apoptosis was observed with culture. Intratubular cell apoptosis was comparable between fresh and cryopreserved cultured fragments and independent of the culture media tested (Fig. 4).

Maturation status of Sertoli cells during testicular culture

Sertoli cell maturation status was verified by AMH and AR immunohistochemical expression.

In the pubertal patient OCID28 (Johnsen score 6), AMH expression remained undetectable in culture. If expressed in testicular tissues before culture (Table I), a statistically significant decrease in the number of tubules containing AMH-expressing Sertoli cells was observed during culture (Fig. 5). However, testicular tissue culture never promoted AR expression by Sertoli cells (data not shown). In fact, when AR was expressed in testicular tissues before culture (OCID23 and OCID28), this expression was lost during the first week of organ culture (data not shown). The Sertoli cell AMH and AR expression patterns in organ culture were independent of FSH/LH addition to the culture medium and identical between fresh and cryopreserved fragments.

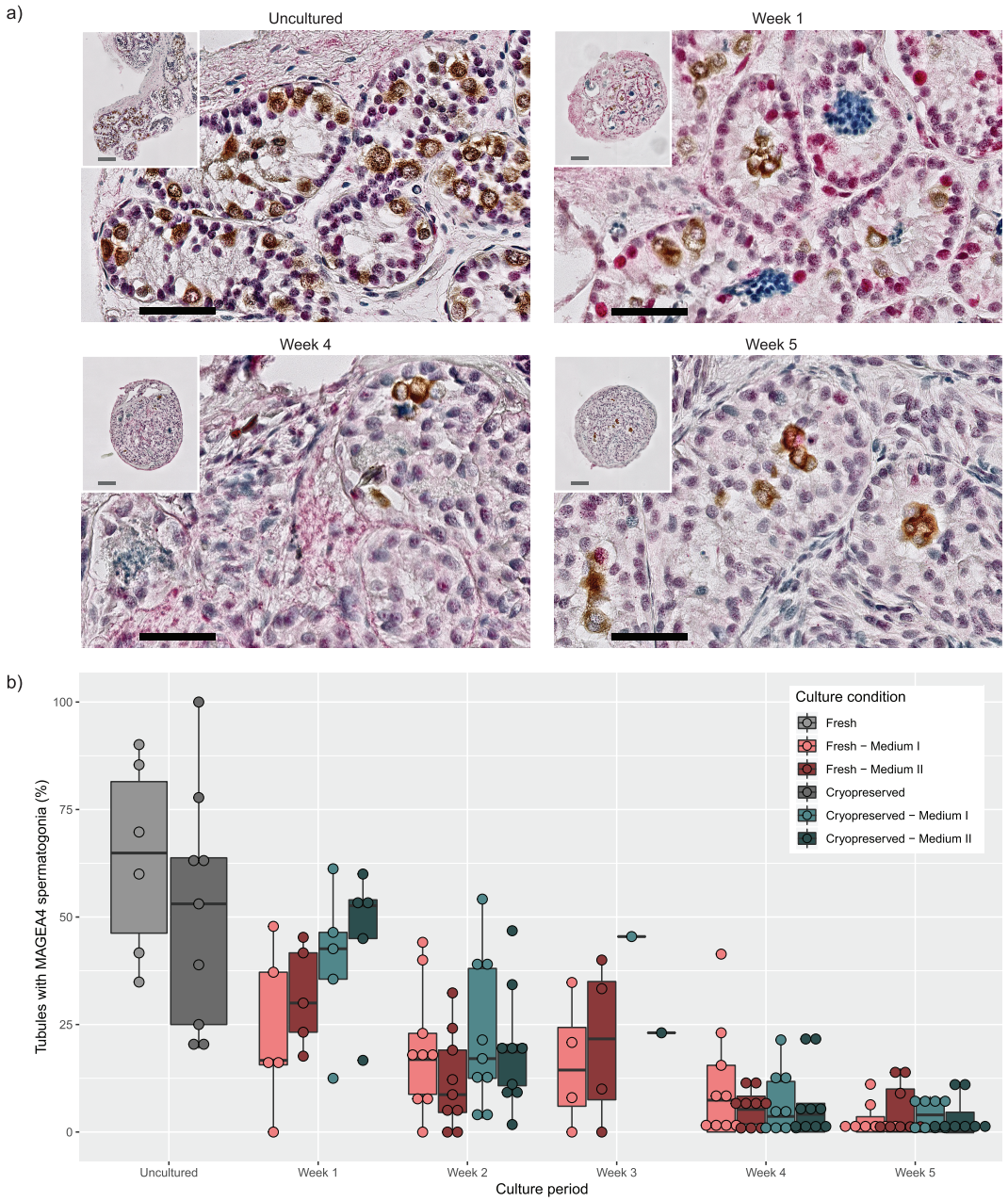

Figure 2 Spermatogonia present in testicular fragments cultured for 5 weeks. (a) Immunohistological identification of MAGEA4 (brown) spermatogonia in a MAGEA4/PCNA (pink) double staining. Scale bars: black = 50 µm; grey = 100 µm. **(b)** Percentage of seminiferous tubules containing at least one MAGEA4-positive cell in fresh (pink) and cryopreserved (blue) testicular fragments cultured in medium without (medium I; faint colour) or with FSH/LH supplementation (medium II; dark colour). Single dots represent results from individual patients, and the boxplot displays the median and 25th and 75th percentiles of all patients at each time point in culture. Mixed-effect linear model revealed a statistically significant reduction ($P \leq 0.001$) during culture.

Testosterone production *in vitro*

Testosterone levels, indicating the functionality of Leydig cells present in cultured testicular fragments, showed a significant increase in culture ($P \leq 0.02$), particularly from culture week 1 to week 3 ($P \leq 0.05$), followed by a stabilization from week 4 onwards (Fig. 6). Testosterone concentrations in medium supplemented with FSH/LH showed a trend towards higher values, but these were not statistically different. Furthermore, for the overall culture period, no statistical difference was observed between testosterone production levels from fresh and cryopreserved cultured fragments. Interestingly, for the pubertal patient with complete spermatogenesis (OCID28), any of the culture conditions sustained Leydig cells' functionality, as testosterone was constantly maintained at low levels (ranging from 0.12 and 1.9 ng/mL) during the complete culture period (Supplementary Fig. S1c).

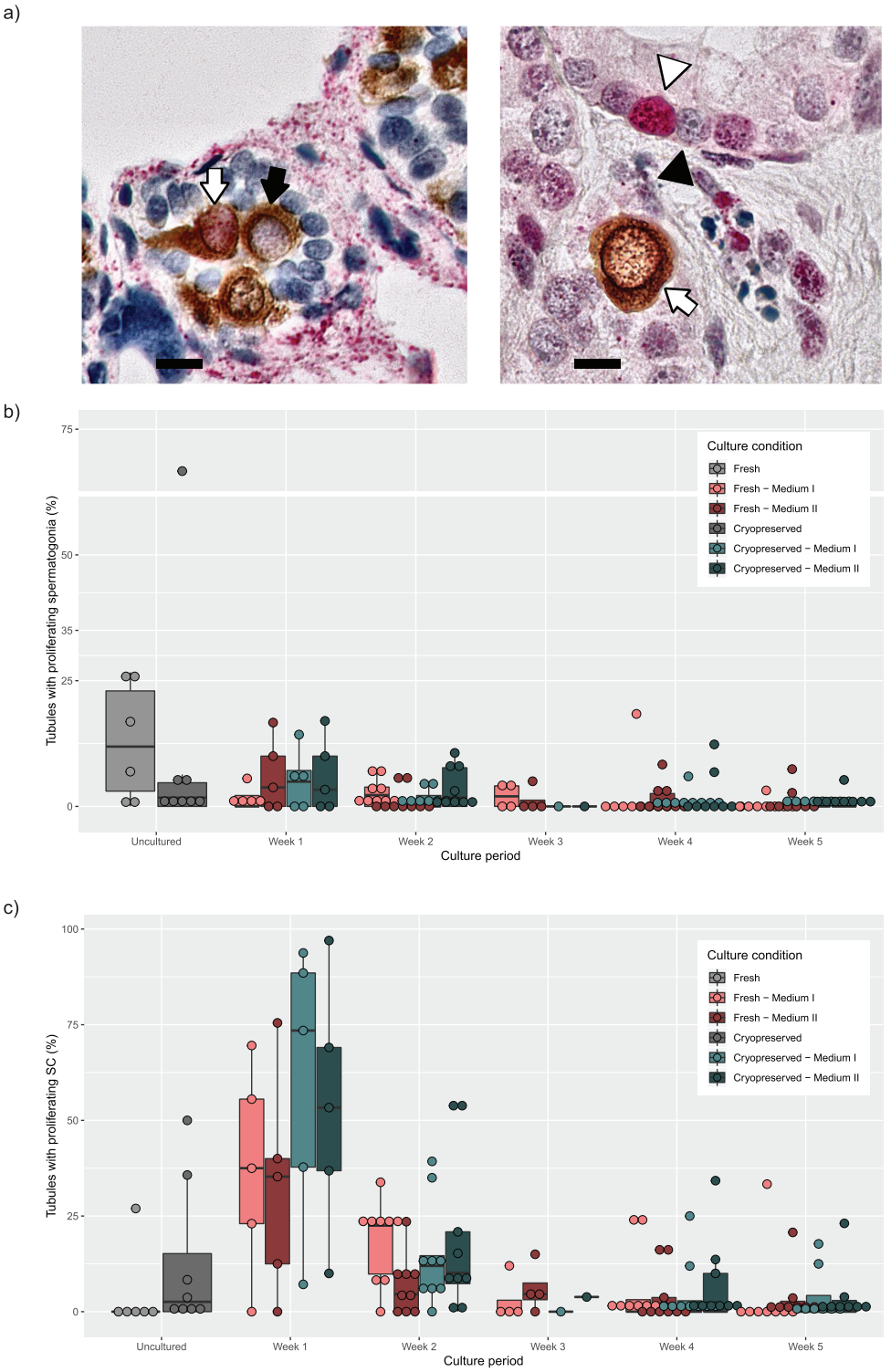

Figure 3 Proliferation status of spermatogonia and Sertoli cells in testicular fragments cultured for 5 weeks. (a) Double immunohistochemical staining of MAGEA4 and PCNA. Proliferative spermatogonia were identified by simultaneous expression of MAGEA4 and PCNA while proliferative Sertoli cells (SCs) were morphologically identified. Black arrow, PCNA-negative spermatogonia; white arrow, PCNA-positive spermatogonia; black arrowhead, PCNA-negative SCs; white arrowhead, PCNA-positive SCs. Scale bar = 10 μm. Percentage of seminiferous tubules containing at least one proliferative (**b)** spermatogonia or (**c)** SCs in fresh (pink) and cryopreserved (blue) testicular fragments cultured in medium without (medium I; faint colour) or with FSH/LH supplementation (medium II; dark colour). Single dots represent results from individual patients, and the boxplot displays the median and 25th and 75th percentiles of all patients at each time point in culture. Mixed-effect linear model revealed a statistically significant reduction ($P \leq 0.001$) during culture.

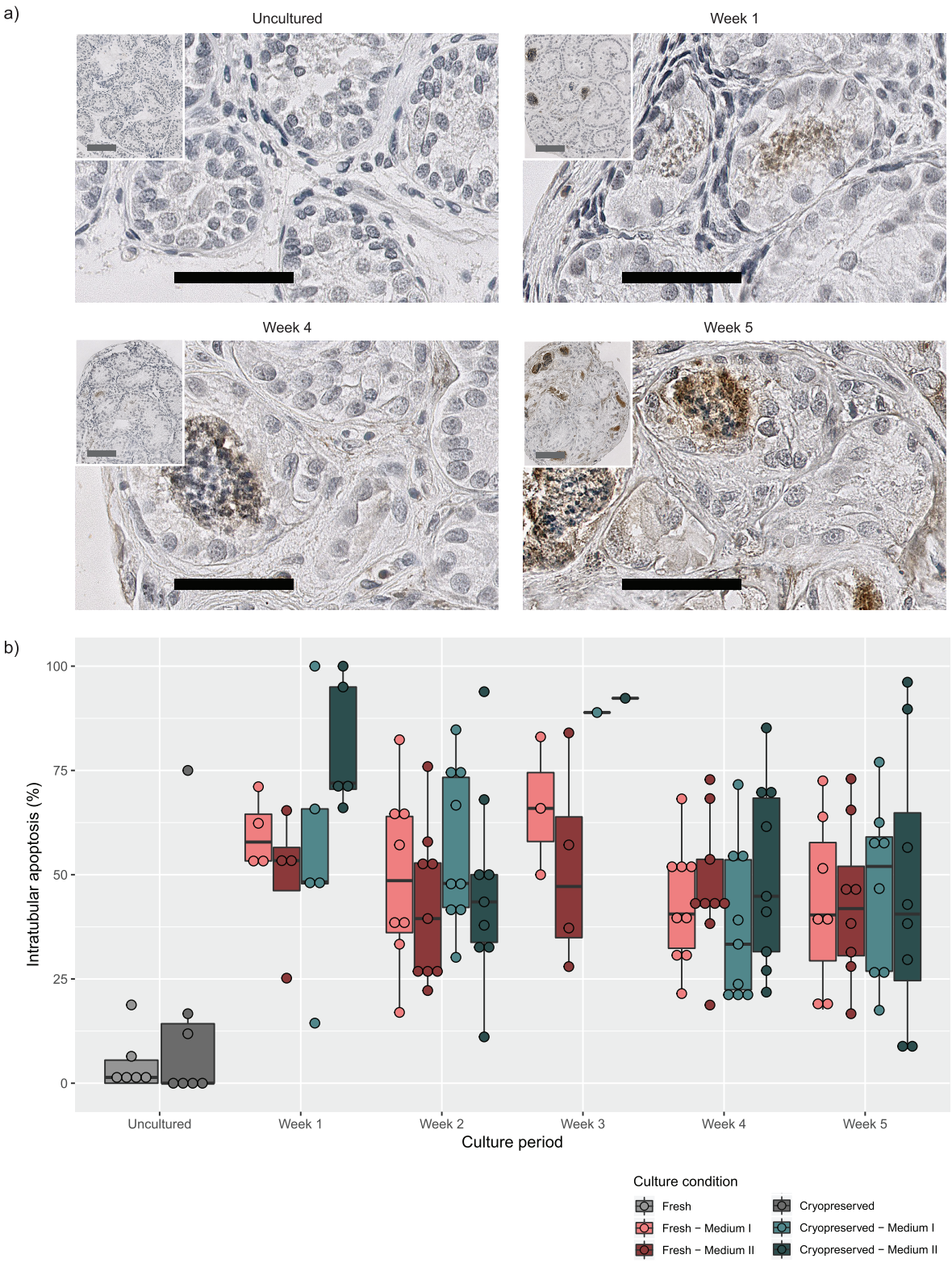

Figure 4 Intratubular cell apoptosis in testicular fragments cultured for 5 weeks. (a) TUNEL staining (brown). Scale bars: black = 50 µm; grey = 100 µm. **(b)** Percentage of intratubular cell apoptosis in fresh (pink) and cryopreserved (blue) testicular fragments cultured in medium without (medium I; faint colour) or with FSH/LH supplementation (medium II; dark colour). Single dots represent results from individual patients, and the boxplot displays the median and 25th and 75th percentiles of all patients at each time point in culture. Mixed-effect linear model revealed a statistically significant increase ($P \leq 0.001$) during culture.

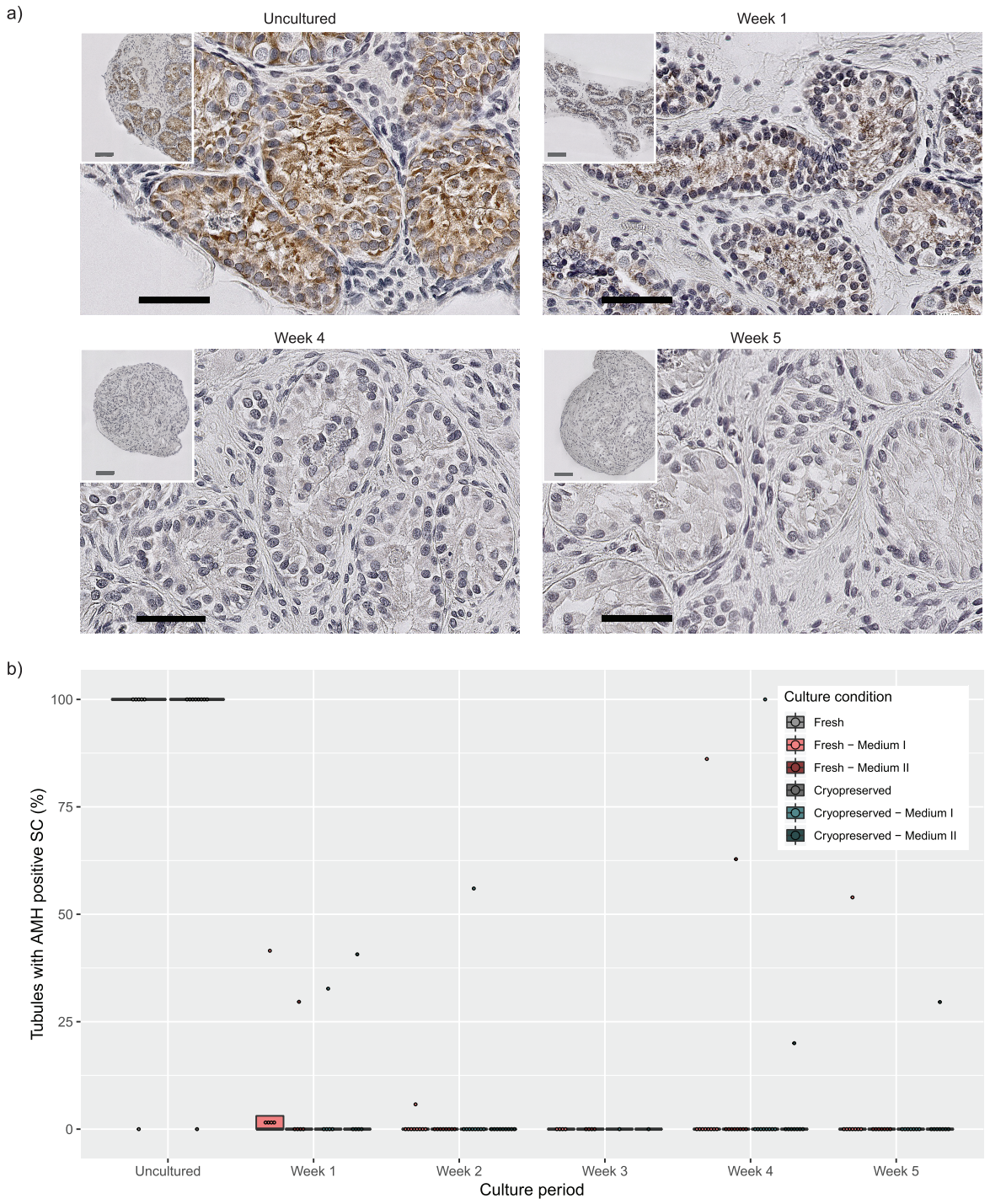

Figure 5 Maturation status of Sertoli cells present in testicular fragments cultured for 5 weeks. (a) AMH (brown) immunohistochemistry. Scale bars: black = 50 µm; grey = 100 µm. **(b)** Percentage of seminiferous tubules containing AMH-positive SCs in fresh (pink) and cryopreserved (blue) testicular fragments cultured in medium without (medium I; faint colour) or with FSH/LH supplementation (medium II; dark colour). Single dots represent results from individual patients, and the boxplot displays the median and 25th and 75th percentiles of all patients at each time point in culture. Mixed-effect linear model revealed a statistically significant reduction ($P \leq 0.001$) during culture.

Discussion

Although attempts to achieve *in vitro* spermatogenesis using cryopreserved human prepubertal testicular tissue have been reported (de Michele *et al.*, 2017, 2018b; Medrano *et al.*, 2018), to our knowledge this is the first report evaluating and comparing the functionality of fresh and cryopreserved testicular fragments in organ culture. *In vitro* initiation of spermatogenesis was not achieved in either

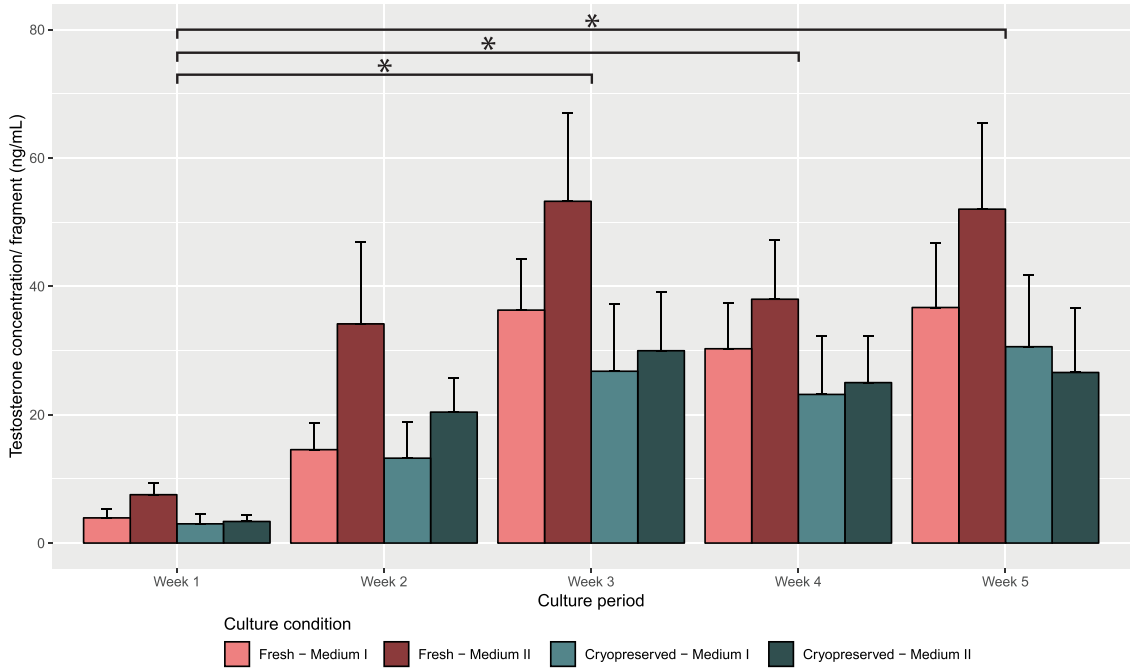

Figure 6 Concentration (ng/mL) of testosterone secreted by fresh (pink) and cryopreserved (blue) testicular fragments during 5 weeks organ culture in medium without (medium I; faint colour) or with FSH/LH supplementation (medium II; dark colour). Data are presented as mean ± SE and $^*P < 0.05$.

fresh or cryopreserved immature testicular tissue in two different media conditions. Our data do suggest that fresh and cryopreserved prepubertal testicular fragments have comparable functionality in the described culture conditions, as reflected by the absence of significant differences in any of the weekly evaluated functional parameters: spermatogonial survival and proliferation, intratubular cell apoptosis, Sertoli cell maturation and proliferation and testosterone production by Leydig cells.

An indication of where a potential improvement might lie in developing the *in vitro* testicular organ culture system for the human tissue comes from two recent papers (de Michele *et al.*, 2018b; Medrano *et al.*, 2018). de Michele and colleagues reported the development of meiotic and postmeiotic cells after 16 days in organ culture of human cryopreserved testicular tissues (de Michele *et al.*, 2018b). In their experiments, the culture media was supplemented with a physiological serum concentration of FSH and the authors claimed that this was essential for meiosis initiation in immature human cryopreserved testicular organ culture (de Michele *et al.*, 2017, 2018b). Likewise, in an FSH and LH supplemented media, Medrano *et al.* observed signs of spermatogenesis initiation followed by a block in maturation at a premeiotic stage (Medrano *et al.*, 2018).

Different outcomes observed between previous studies and ours might be explained by the culture media composition (compared in Supplementary Table SV). Furthermore, the use of different cryopreservation protocols, involvement of distinct cohorts including patients with various initial maturation states, ages and different cancer diagnoses with unknown consequences for testicular function and differentiation potential *in vitro* are important factors to consider when explaining the differences in outcome between studies. Nevertheless, complete spermatogenesis was never achieved and progressive germ cell loss was evident in all studies (de Michele *et al.*, 2018b; Medrano *et al.*, 2018). Therefore, the functionality and ability of cryopreserved human prepubertal testicular tissue to complete spermatogenesis remain to be demonstrated.

In our study, some spermatogonia survived and remained proliferative active in all culture conditions; however, a substantial decrease of the spermatogonial numbers was observed over culture time, possibly justified by the combination of reduced proliferation activity and increased intratubular cell apoptosis. This was accompanied by a failed induction or maintenance (depending on maturation status before culture) of Sertoli cell maturation and a transient increase in Sertoli cell proliferative activity *in vitro*. While a decline in AMH expression suggested maturation of Sertoli cells *in vitro*, the culture conditions tested in this study did not support normal maturation of Sertoli cells as evidenced by the absence of AR expression. Discordant indications of Sertoli cells maturation status demonstrated by proliferative activity, AMH and AR expression might be suggestive of Sertoli cell dysfunction (Sharpe *et al.*, 2003). Similar observations were previously reported demonstrating a reduction in AMH expression and secretion, while AR expression results were inconclusive (de Michele *et al.*, 2017, 2018b; Medrano *et al.*, 2018). Furthermore, establishment of a blood–testis barrier by adjacent Sertoli cells (indicative of differentiated state) was reported to be solely partially achieved or maintained in organ culture as revealed by expression analysis of tight and gap junctions proteins (de Michele *et al.*, 2018a).

These results suggest that the current organ culture conditions are unable to support correct Sertoli cell maturation in prepubertal testicular tissue, a consequence of which might be germ cells loss and

incapability to support complete progression of human spermatogenesis (Jørgensen *et al*., 2018).

Since the recovery of fertility potential of cryopreserved tissue has still not been proven in humans, the cryopreservation protocol remains experimental and may require improvements to ensure the ability of tissue to complete spermatogenesis by organ culture (Picton *et al.*, 2015). Although no differences between fresh and cryopreserved testicular tissue were noticeable in this study, we cannot exclude that if an optimized culture method ensuring complete spermatogenesis is established, functional or spermatogenic efficiency differences between fresh and cryopreserved tissue might be found.

A direct translation of the organ culture conditions established in mice to other animal models failed to result in identical spermatogenic outcomes. In fact, further adaptations of the culture media have not yet resulted in full spermatogenesis from immature spermatogonia in rats nor in humans (Reda *et al.*, 2016; de Michele *et al.*, 2017, 2018b; Medrano *et al.*, 2018). Attempting to better mimic the human pubertal testicular niche *in vivo* by stimulating spermatogonia self-renewal and differentiation as well as somatic cells maturation, we tried to further refine the culture media with retinoic acid and melatonin (medium I) as well as additional supplementation with FSH and LH (medium II). *In vivo* and *in vitro* studies in rodents have shown that retinoic acid is an essential factor for germ cell differentiation and maintenance (van Pelt and de Rooij, 1991; Koubova *et al.*, 2014; Arkoun *et al.*, 2015) and melatonin is known to prevent tissue damage induced by reactive oxygen or nitrogen species due to cytotoxic exposure (Madhu *et al.*, 2016; Frungieri *et al.*, 2017). Although the testicular architecture was maintained in cultured testicular fragments, medium I (containing retinoic acid and melatonin) was not enough to stimulate initiation of spermatogenesis *in vitro*. FSH and LH are gonadotrophins known to play a key role in testicular maturation and germ cell development. While LH acts on Leydig cells stimulating testosterone production and consequently inducing testicular maturation, FSH supports spermatogenesis via effects on Sertoli cells metabolism (Ramaswamy and Weinbauer, 2014). Our results show that, for all of the functional parameters (spermatogonial survival and proliferation, intratubular cell apoptosis, Sertoli cell maturation, and proliferation) evaluated on fresh and cryopreserved cultured testicular fragments, medium supplementation with LH and FSH (medium II) did not induce significant improvements compared to non-supplemented medium (medium I). Even so, Leydig cells endocrine function was stimulated in all culture conditions, with no difference reflected in testosterone production in medium supplemented with FSH/LH (medium II) compared to non-supplemented medium (medium I).

The initial maturation status of the testicular tissues might also have considerable influence on the efficiency of the organ culture. In fact, Sato *et al.* clearly described lower spermatogenic efficiency of organ culture of adult mouse testicular fragments when compared to neonatal tissue (Sato *et al.*, 2015). Although not statistically confirmed, Medrano *et al.* observed a lower number of conserved testicular tubules after organ culture from samples with a more advanced maturation stage (Johnsen score 6–8), suggesting a higher sensitivity of the testicular tissue at a more advanced stage of spermatogenesis to *in vitro* conditions (Medrano *et al.*, 2018). In our study, the histological maturation state of the testicular tissues was homogenous with spermatogonia as the most advanced germ cell type, except for one patient who had complete spermatogenesis. Interestingly, and in accordance with the literature, in the pubertal patient (OCID28; Johnsen score 6), the somatic environment was greatly affected by culture as observed by the extremely low concentration of testosterone production and increased tissue sclerosis. However, due to the limited number of patients with complete spermatogenesis included in this study, further comprehensive analysis should be done to confirm this hypothesis.

The different steps of germ cell development are dependent on the appropriate hormonal environment that fluctuates through (pre)puberty. In this study, a single and constant concentration of gonadotropins was used for all patients, independently of their initial maturation status. In fact, it might be that these tissues have different hormonal requirements and culture conditions depending on patients' testicular development.

Concluding, we reported that the current testicular organ culture system supports, both for fresh and for cryopreserved tissue, maintenance of the testicular architectural structure, spermatogonial proliferation and endocrine function but does not induce spermatogenesis. As testicular cryopreservation is actively offered to prepubertal cancer patients, this study highlights the necessity for further improvement of the current organ culture method to become clinically applicable, ensuring better spermatogonial survival as well as differentiation to sperm in cryopreserved testicular biopsies. Furthermore, the current inability to initiate spermatogenesis *in vitro* from cryopreserved testicular fragments should form part of the counselling of patients included in fertility preservation programmes.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgments

We thank Dr O.J. de Boer from the Department of Pathology (AMC), for support with the Philips IntelliSite UFS slide scanner, J. Eliveld from the Center of Reproductive Medicine (AMC), for assistance with testosterone measurements, and Dr M. van Wely from the Center of Reproductive Medicine (AMC), for advice on statistical analysis.

Authors' roles

This study was designed by J.M.D.P., A.M.M.P. and S.R. Testicular tissue was biopsied by A.M. and cryopreserved by A.A.M. Experiments were done by J.M.D.P., S.K.M.D. and C.M.W.K. J.M.D.P. and A.M.M.P contributed for data acquisition, analysis and interpretation and drafting of the manuscript. All authors critically revised the manuscript and approved the final version.

Funding

Growsperm (EU-FP7-PEOPLE-2013-ITN 603568).

Conflict of interest

None of the authors have any competing interests to declare.

References

Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WHB. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. *Lancet Diabetes Endocrinol* 2015;**3**:556–567.

Arkoun B, Dumont L, Milazzo JP, Way A, Bironneau A, Wils J, Macé B, Rives N. Retinol improves in vitro differentiation of pre-pubertal mouse spermatogonial stem cells into sperm during the first wave of spermatogenesis. *PLoS One* 2015;**10**:1–23.

Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci U S A* 1994;**91**:11303–11307.

Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 1972;**52**:198–236.

de Michele F, Poels J, Giudice MG, De Smedt F, Ambroise J, Vermeulen M, Gruson D, Wyns C. In vitro formation of the blood-testis barrier during long-term organotypic culture of human prepubertal tissue: comparison with a large cohort of pre/peripubertal boys. *Mol Hum Reprod* 2018a;**24**:271–282.

de Michele F, Poels J, Vermeulen M, Ambroise J, Gruson D, Guiot Y, Wyns C. Haploid germ cells generated in organotypic culture of testicular tissue from prepubertal boys. *Front Physiol* 2018b;**9**:1–18.

de Michele F, Poels J, Weerens L, Petit C, Evrard Z, Ambroise J, Gruson D, Wyns C. Preserved seminiferous tubule integrity with spermatogonial survival and induction of Sertoli and Leydig cell maturation after long-term organotypic culture of prepubertal human testicular tissue. *Hum Reprod* 2017;**32**:32–45.

Dumont L, Arkoun B, Jumeau F, Milazzo JP, Bironneau A, Liot D, Wils J, Rondanino C, Rives N. Assessment of the optimal vitrification protocol for pre-pubertal mice testes leading to successful in vitro production of flagellated spermatozoa. *Andrology* 2015;**3**:611–625.

Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, Houser L, Robertson N, Roberts V, Ramsey C *et al.* Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. *Science* 2019;**363**:1314–1319.

Frungieri MB, Calandra RS, Rossi SP. Local actions of melatonin in somatic cells of the testis. *Int J Mol Sci* 2017;**18**:E1170.

Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G *et al.* Regenerates spermatogenesis producing functional sperm. *Cell Stem Cell* 2012;**11**:715–726.

Honaramooz A, Li M-W, Penedo MCT, Meyers S, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice. *Biol Reprod* 2004;**70**:1500–1503.

Honaramooz A, Snedaker A, Boiani M, Schöler H, Dobrinski I, Schlatt S. Sperm from neonatal mammalia testes grafted in mice. *Nature* 2002;**418**:778–781.

Jahnukainen K, Ehmcke J, Hou M, Schlatt S. Testicular function and fertility preservation in male cancer patients. *Best Pract Res Clin Endocrinol Metab* 2011;**25**:287–302.

Jan SZ, Vormer TL, Jongejan A, Röling M, Silber SJ, de Rooij DG, Hamer G, Repping S, van Pelt AMM. Unraveling transcriptome dynamics in human spermatogenesis. *Development* 2017;**144**:3659–3673.

Johnsen SG. Testicular biopsy score count-a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* 1970;**1**:2–25.

Jørgensen A, Macdonald J, Nielsen JE, Kilcoyne KR, Perlman S, Lundvall L, Langhoff Thuesen L, Juul Hare K, Frederiksen H, Andersson AM *et al.* Nodal signaling regulates germ cell development and establishment of seminiferous cords in the human fetal testis. *Cell Rep* 2018;**25**:1924–1937.

Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. *Hum Reprod* 2007;**22**:1384–1395.

Koubova J, Hu YC, Bhattacharyya T, Soh YQS, Gill ME, Goodheart ML, Hogarth CA, Griswold MD, Page DC. Retinoic acid activates two pathways required for meiosis in mice. *PLoS Genet* 2014;**10**:e1004541.

Liu Z, Nie YH, Zhang CC, Cai YJ, Wang Y, Lu HP, Li YZ, Cheng C, Qiu ZL, Sun Q. Generation of macaques with sperm derived from juvenile monkey testicular xenografts. *Cell Res* 2016;**26**:139–142.

Madhu P, Reddy KP, Reddy PS. Role of melatonin in mitigating chemotherapy-induced testicular dysfunction in Wistar rats. *Drug Chem Toxicol* 2016;**39**:137–146.

Medrano JV, Vilanova-Pérez T, Fornés-Ferrer V, Navarro-Gomezlechon A, Martínez-Triguero ML, García S, Gómez-Chacón J, Povo I, Pellicer A, Andrés MM *et al.* Influence of temperature, serum, and gonadotropin supplementation in short- and long-term organotypic culture of human immature testicular tissue. *Fertil Steril* 2018;**110**:1045–1057.

Meistrich ML. The effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertil Steril* 2013;**100**:1180–1186.

Ntemou E, Kadam P, Van Saen D, Wistuba J, Mitchell RT, Schlatt S, Goossens E. Complete spermatogenesis in intratesticular testis tissue xenotransplants from immature non-human primate. *Hum Reprod* 2019;**34**:403–413.

Phillips SM, Padgett LS, Leisenring WM, Stratton KK, Bishop K, Krull KR, Alfano CM, Gibson TM, de Moor JS, Hartigan DB *et al.* Survivors of childhood cancer in the United States: prevalence and burden of morbidity. *Cancer Epidemiol Biomarkers Prev* 2015;**24**:653–663.

Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H *et al.* A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. *Hum Reprod* 2015;**30**:2463–2475.

Ramaswamy S, Weinbauer GF. Endocrine control of spermatogenesis: role of FSH and LH/testosterone. *Spermatogenesis* 2014;**4**:e996025.

Reda A, Albalushi H, Montalvo SC, Nurmio M, Sahin Z, Hou M, Geijsen N, Toppari J, Söder O, Stukenborg JB. Knock-out serum replacement and melatonin effects on germ cell differentiation in murine testicular explant cultures. *Ann Biomed Eng* 2017;**45**:1783–1794.

Reda A, Hou M, Winton TR, Chapin RE, Söder O, Stukenborg JB. In vitro differentiation of rat spermatogonia into round spermatids in tissue culture. *Mol Hum Reprod* 2016;**22**:601–612.

Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature* 2011a;**471**:504–508.

Sato T, Katagiri K, Kojima K, Komeya M, Yao M, Ogawa T. In vitro spermatogenesis in explanted adult mouse testis tissues. *PLoS One* 2015;**10**:1–13.

Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, Matoba S, Ogura A, Ogawa T. In vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nat Commun* 2011b; **2**:472–477.

Schrader M, Müller M, Straub B, Miller K. The impact of chemotherapy on male fertility: a survey of the biologic basis and clinical aspects. *Reprod Toxicol* 2001;**15**:611–617.

Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* 2003;**125**: 769–784.

Shetty G, Mitchell JM, Lam TNA, Wu Z, Zhang J, Hill L, Tailor RC, Peters KA, Penedo MC, Orwig KE *et al.* Donor spermatogenesis in de novo formed seminiferous tubules from transplanted testicular cells in rhesus monkey testis. *Hum Reprod* 2018;**33**: 2249–2255.

Stukenborg J-B, Alves-Lopes JP, Kurek M, Albalushi H, Reda A, Keros V, Töhönen V, Bjarnason R, Romerius P, Sundin M *et al.* Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy. *Hum Reprod* 2018;**33**:1677–1683.

Wallace WHB. Oncofertility and preservation of reproductive capacity in children and young adults. *Cancer* 2011;**117**:2301–2310.

van Pelt AMM, de Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. *Endocrinology* 1991;**128**:697–704.

Yokonishi T, Sato T, Komeya M, Katagiri K, Kubota Y, Nakabayashi K, Hata K, Inoue K, Ogonuki N, Ogura A *et al.* Offspring production with sperm grown in vitro from cryopreserved testis tissues. *Nat Commun* 2014;**5**:1–6.