

DNA methylation and histone post-translational modifications in the mouse germline following in-vitro maturation of fresh or cryopreserved prepubertal testicular tissue

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A. Oblette, J. Rondeaux, L. Dumont, M. Delessard, J. Saulnier, et al.. DNA methylation and histone post-translational modifications in the mouse germline following in-vitro maturation of fresh or cryopreserved prepubertal testicular tissue. 2019, 39 (3), pp.383-401. 10.1016/j.rbmo.2019.05.007. hal-02301873

HAL Id: hal-02301873 https://hal.science/hal-02301873

Submitted on 20 Jul2022

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1	Running title: Epigenetic modifications in <i>in vitro</i> cultured mouse testes
2	
3	Title: DNA methylation and histone post-translational modifications in the mouse germline
4	following in vitro maturation of fresh or frozen/thawed prepubertal testicular tissue
5	
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18 Abstract

20	Research question: Do freezing/thawing and in vitro culture procedures affect the expression
21	of DNA methyltransferases (DNMTs) and histone modifying enzymes, as well as the
22	establishment of DNA methylation and histone post-translational modifications (PTMs) in
23	germ cells in prepubertal mouse testicular tissue?
24	Design: We investigated the expression of epigenetic modification enzymes, DNA
25	methylation and histone PTMs, and the spermatogenic progression after in vitro maturation of
26	fresh or frozen/thawed mouse prepubertal testicular tissue. Fresh or thawed testicular
27	fragments from 6-7 days <i>postpartum</i> mice were cultured for 30 days in the presence of retinol
28	with or without follicle stimulating hormone (FSH).
29	Results: The <i>in vitro</i> maturation of fresh or frozen/thawed tissue allowed the differentiation
30	of spermatogonia into spermatozoa. Differences in the levels of transcripts encoding
31	epigenetic modifications enzymes (Dnmt1, Dnmt3a, Prdm9, Jarid1b, Src1, Sirt1, Hdac1)
32	were found between 30-day tissue cultures and age-matched in vivo controls.
33	DNMT1/DNMT3a expression and the presence of 5-methylcytosine (5mC) were detected in
34	spermatogonia and leptotene/zygotene spermatocytes in cultures. The relative 5mC
35	fluorescence intensity was similar in spermatozoa produced in cultures of frozen/thawed
36	tissues or in vivo. H3K4me3, H3K9ac and H4K8ac were present in all germ cell types but
37	differences in the proportion of germ cells containing these epigenetic marks were found after
38	cultures.
39	Conclusions: Despite differences with the <i>in vivo</i> situation, DNA methylation and histone
40	methylation and acetylation occur in the mouse germline in <i>in vitro</i> matured fresh or
41	frozen/thawed mouse prepubertal testicular tissue, and the expression of the enzymes
42	catalyzing these epigenetic modifications are maintained in vitro.

44	Keywords: mouse prepubertal testis, freezing protocols, in vitro spermatogenesis, epigenetic
45	modification, DNA methylation, histone post-translational modifications.
46	
47	Key message: In prepubertal boys with cancer, fertility preservation relies on testicular tissue
48	freezing. In vitro maturation of frozen/thawed tissue is one of the approaches envisaged to
49	restore fertility. The impact of the procedures on germline epigenetic modifications needs to
50	be evaluated in an animal model before considering a human application.

51 Introduction

52

53	Survival rates for children with cancer have considerably increased over the past decades
54	thanks to diagnostic and therapeutic progress. Treatments by chemotherapy and radiotherapy
55	can however have toxic effects on gonads (Jahnukainen et al., 2015; Picton et al., 2015; Wyns
56	et al., 2010). In prepubertal boys, the actively dividing spermatogonial stem cells (SSCs) are
57	particularly sensitive to certain cytotoxic agents used in chemotherapy protocols (Goossens et
58	al., 2013; Nurmio et al., 2009). At adulthood, cured patients can be diagnosed with a
59	permanent infertility (oligozoospermia, azoospermia) with significant psychological
60	consequences (Gies et al., 2015; Jahnukainen et al., 2015).
61	Because of the potential long-term sequelae of cancer treatment on reproductive functions,
62	fertility preservation options have been developed and are proposed to patients prior to cancer
63	therapy (Baert et al., 2012; Gies et al., 2015; Jahnukainen et al., 2015; Onofre et al., 2016;
64	Picton et al., 2015; Wyns et al., 2015). For adolescents and adults, sperm can be
65	cryopreserved before the beginning of gonadotoxic treatments and later used for assisted
66	reproductive techniques. For prepubertal boys however, freezing the testicular tissue that
67	contains SSCs is so far the only option. Freezing (slow freezing or vitrification) and thawing
68	procedures have been optimized for immature testicular tissues in order to minimize ice
69	crystal formation and cellular damage (Baert et al., 2012; Poels et al., 2013). We previously
70	reported the development of optimal controlled slow freezing (CSF) and solid surface
71	vitrification (SSV) protocols that preserve not only the architecture of mouse prepubertal
72	tissue but also its exocrine and endocrine functions (Arkoun et al., 2016; Dumont et al., 2015;
73	Milazzo et al., 2008).
74	In order to restore the fertility of cancer survivors who became sterile, thawed testicular

tissues could theoretically be matured by *in vivo* or *in vitro* approaches to produce

76 spermatozoa for assisted reproduction (de Michele et al., 2017; Gies et al., 2015; Ibtisham et 77 al., 2017; Yokonishi and Ogawa, 2016). The in vivo differentiation of SSCs could be achieved 78 after autologous transplantation or autograft or eventually after xeno-transplantation or 79 xenograft (Goossens et al., 2013; Milazzo et al., 2010; Poels et al., 2013). To avoid the 80 potential reintroduction of malignant cells to surviving patients during auto-transplantation of 81 cryopreserved testicular tissue or cells, especially to those treated for lymphoblastic acute 82 leukemia, testicular tissues could be matured *in vitro* (Jahnukainen et al., 2015). 83 Tridimensional and organotypic cultures are so far the only methods that allowed the 84 production of spermatozoa from prepubertal murine testes (Abu Elhija et al., 2012; Arkoun et 85 al., 2016; Dumont et al., 2015; Sato et al., 2011; Yokonishi et al., 2014). Notably, a complete 86 in vitro spermatogenesis could be achieved in frozen (slow freezing or vitrification)/thawed 87 mouse prepubertal testicular tissues cultured at a gas-liquid interphase (Arkoun et al., 2016; 88 Dumont et al., 2015; Yokonishi et al., 2014). Our data moreover showed that supplementing the culture medium with 10⁻⁶ M retinol promoted SSCs differentiation and entry into meiosis 89 90 as well as sperm production in fresh or frozen/thawed testes (Arkoun et al., 2015; Dumont et 91 al., 2017, 2016; Rondanino et al., 2017). A recent study confirmed that retinoic acid and 92 retinol play crucial roles in the onset of spermatogonial differentiation and meiotic initiation 93 under organ culture conditions (Sanjo et al., 2018). The addition of follicle stimulating 94 hormone (FSH), crucial for the initiation and progression of spermatogenesis, together with 95 retinol in the medium had no additive effect on the spermatogenic yield (Arkoun et al., 2015). 96 We recently reported that most of *in vitro* generated spermatozoa were haploid, contained 97 unfragmented DNA and normally condensed chromatin like their in vivo counterparts 98 (Oblette et al., 2017). Although the microinjection of *in vitro* produced spermatozoa into 99 oocytes was capable of producing reproductively competent offspring (Sato et al., 2011; 100 Yokonishi et al., 2014) with an almost normal state of methylation for 11 imprinted genes

101 (Yokonishi et al., 2014), the epigenetic modifications of germ cells in cultured prepubertal 102 testes have never been studied. Epigenetic marks, including DNA methylation and histone post-translational modifications (PTMs), are however important during spermatogenesis for 103 104 the proper maturation of gametes, and after fertilization the inherited marks play an essential 105 role during embryonic development and can later influence offspring health and behavior 106 (Champroux et al., 2018; Jenkins et al., 2017; Stewart et al., 2016; Stuppia et al., 2015; Yao et 107 al., 2015; Boissonnas et al., 2013). DNA methylation is an epigenetic mechanism involving 108 the covalent transfer of a methyl group onto the C5 position of cytosine to form the heritable 109 mark 5-methylcytosine (5mC). This epigenetic modification, which is catalyzed by DNA 110 methyltransferases (DNMT), represses the expression of numerous genes. DNMT1 is 111 responsible for the maintenance of DNA methylation profiles during cell divisions (Uysal et 112 al., 2016). DNMT3a and DNMT3b catalyze *de novo* DNA methylation in a heterodimer with 113 the non-catalytic DNMT3L (Uysal et al., 2016). During mouse development, the genome of 114 primordial germ cells undergoes a wave of demethylation between 8 and 13.5 days post 115 coïtum (Boissonnas et al., 2013). De novo methylation marks are then established in a sex-116 specific manner. In males, de novo DNA methylation to establish paternally imprinted loci 117 begins at around 15.5 days post coïtum and continues after birth in spermatogonia and 118 spermatocytes. The process of *de novo* DNA methylation appears to be complete at the 119 pachytene spermatocyte stage (Boissonnas et al., 2013; Yao et al., 2015). Knocking out the 120 mouse Dnmt1 gene was shown to induce embryonic lethality, and disruption of Dnmt3a and 121 Dnmt3l genes led to impaired spermatogenesis (Jenkins and Carrell, 2012; Uysal et al., 2016). 122 In men, abnormal sperm DNA methylation patterns have been associated with infertility, poor 123 embryo quality and congenital diseases in the offspring (Cui et al., 2016; Gunes et al., 2016; 124 Kitamura et al., 2015; McSwiggin and O'Doherty, 2018; Santi et al., 2017; Stuppia et al., 125 2015). Histone PTMs, such as methylation and acetylation, have been reported to be

126 associated with chromatin remodeling during spermatogenesis, including histone-to-127 protamine replacement occurring within germ cells during spermiogenesis. Histone 128 methylation corresponds to the addition of one or several methyl groups on lysine (K) or 129 arginine (R) residues by histone methyltransferases (e.g. PRDM9) and can be reversed by 130 histone demethylases (e.g. JARID1B). PRDM9, which catalyzes the trimethylation of lysine 4 131 of histone H3 (H3K4me3), is essential for progression through early meiotic prophase, 132 including homologous chromosome pairing, DNA double-strand break repair and sex body 133 formation (Hayashi et al., 2005). This mark is associated with transcriptionally active genes 134 and is also enriched at developmental gene promoters within spermatozoa (Hammoud et al., 135 2011). The acetylation of histones on lysine residues, an epigenetic modification found in 136 transcriptionally active chromatin regions, is catalyzed by histone acetyltransferases (e.g. 137 SRC1) and reversed by histone deacetylases (e.g. SIRT1, HDAC1). An increase in the 138 acetylation of histones H3 and H4 is observed in spermatogonia and spermatids compared to 139 spermatocytes (Boissonnas et al., 2013; Luense et al., 2016). The H3K9ac mark is essential 140 for the progression of spermiogenesis: alterations in H3K9ac levels induce morphological 141 abnormalities and apoptosis of round spermatids (Dai et al., 2015). Mice with a specific 142 deletion of *Sirt1* in male germ cells exhibit a defect in histone-to-protamine transition, altered 143 chromatin condensation, defects in acrosome biogenesis and decreased fecundity (Bell et al., 144 2014; Liu et al., 2017). Moreover, a premature acetylation of histone H4 in spermatocytes or a 145 decrease of histone acetylation in spermatids have been reported in infertile men exhibiting 146 impaired spermatogenesis (Sonnack et al., 2002). Alterations in the genomic distribution of 147 the heritable marks H3K4me3, H3K9ac and H4K12ac have also been described in the 148 spermatozoa of infertile men (Hammoud et al., 2011; Steilmann et al., 2011; Vieweg et al., 149 2015).

150 It has been demonstrated that DNMT1 and DNMT3A expression levels, DNA methylation 151 status of germ cells and several histone PTMs (H3K4me3, H3K9ac, H4K12ac, H4K16ac) 152 were unchanged after in vivo maturation of SSCs from prepubertal mouse testes by 153 transplantation or graft (Goossens et al., 2011). However, H4K5ac and H4K8ac marks were 154 altered after SSCs transplantation (Goossens et al., 2011). It appears therefore imperative to 155 ascertain that DNA methylation and histone PTMs are not altered in the germline in 156 organotypic cultures before applying *in vitro* maturation procedures to frozen/thawed 157 testicular tissue from prepubescent boys. So far, the maintenance of DNA methylation has 158 only been shown in 2 to 4-day cultures of rat fetal testes (Rwigemera et al., 2017) and in 7 to 159 21-day cultures of marmoset adult testes (Langenstroth-Röwer et al., 2017). Histone PTMs 160 (H3K4me2, H3K4me3) were also maintained in short-term cultures (Rwigemera et al., 2017). 161 In the present work, fresh, CSF or SSV mouse prepubertal testicular tissues were cultured for 162 30 days at a gas-liquid interphase in the presence of retinol alone or of a retinol/FSH 163 alternation. The progression of *in vitro* spermatogenesis was analyzed. DNA methylation, 164 active histone marks (H3K4me3, H3K9ac, H4K8ac) and the expression of catalytic DNMTs 165 and histone modifying enzymes were studied in germ cells prior and following in vitro 166 maturation.

167

168 Materials and Methods

169

170 Mice, testes collection and ethical approval

171 Prepubertal 6-7-day-old CD-1 male mice (Charles River Laboratories, L'Arbresle, France)

172 were euthanized by decapitation. Testes were excised, rinsed and the tunica albuginea was

173 removed in α-MEM at 4°C (Gibco by Life Technologies, Saint-Aubin, France). Testes were

then divided in several batches and cultured either directly (culture of fresh tissues) or after

175 CSF or SSV and thawing (Additional File 1). In order to evaluate the impact of the freezing

176 procedures on the testicular tissues before culture, fresh, CSF and SSV testes from mice aged

177 6-7 dpp were analyzed (Additional File 1). Moreover, mice aged 36-37 dpp were euthanized

by CO₂ asphyxiation and their testes were used as *in vivo* controls for testes cultured *in vitro*

179 for 30 days (Additional File 1). In total, 42 testes (14 fresh, 14 CSF, 14 SSV) from 21 mice

aged 6-7 dpp were used for the analyses performed before culture, 72 testes (24 fresh, 24

181 CSF, 24 SSV) from 39 mice aged 6-7 dpp were used for *in vitro* maturation, and 20 testes

182 from 13 mice aged 36-37 dpp were used as *in vivo* controls. A total of 134 testes from 73

183 mice were therefore used in this study (Additional File 1).

184 This study was conducted in accordance with the 3Rs principle. All the experimental

185 procedures were approved by the Institutional Animal Care and Use Committee of Rouen

186 University Hospital under the licence/protocol number N/23-11-12/46/11-15.

187

188 CSF and thawing of testicular tissues

189 *Freezing procedure.* Testes were frozen as previously described (Arkoun et al., 2016;

190 Milazzo et al., 2008). Briefly, testes were incubated in cryotubes containing 1.3 mL of

191 freezing medium: Leibovitz L-15 medium (Eurobio, Courtabœuf, France) supplemented with

192 1.5 M dimethylsulfoxyde (DMSO, Sigma-Aldrich, Saint-Quentin Fallavier, France), 0.05 M

sucrose (Sigma-Aldrich) and 10% (v/v) fetal calf serum (FCS, Eurobio). Controlled slow

194 freezing was performed in a programmable freezer (Nicool Freezal, Air Liquide, Marne-la-

195 Vallée, France) with the following protocol: start at 5°C, then -2°C/minute until reaching -

196 9°C, stabilization at -9°C for 7 minutes, then -0.3°C/minute until -40°C and -10°C/minute

197 down to -140°C. The samples were then stored in liquid nitrogen.

198 *Thawing procedure.* Cryotubes were warmed for 1 minute at room temperature and then for 3

199 minutes at 30°C in a water bath. Testicular tissues were then successively incubated at 4°C in

- 200 baths containing decreasing concentrations of cryoprotective agents during 5 minutes each
- 201 (bath 1: 1 M DMSO, 0.05 M sucrose, 10% FCS, Leibovitz L15; bath 2: 0.5 M DMSO, 0.05
- 202 M sucrose, 10% FCS, Leibovitz L15; bath 3: 0.05 M sucrose, 10% FCS, Leibovitz L15; bath

203 *4*: 10% FCS, Leibovitz L15).

204

205 SSV and thawing of testicular tissues

- 206 *Vitrification procedure.* Testes were vitrified as previously described (Dumont et al., 2015).
- 207 Briefly, samples were incubated for 10 minutes at 4°C in Dulbecco's modified Eagle's
- 208 medium/Nutrient Mixture F12 (DMEM/F12, Sigma-Aldrich) supplemented with 1.05 M
- 209 DMSO and 1.35 M ethylene glycol (Sigma-Aldrich) and then for 5 minutes at 4°C in
- 210 DMEM/F12 supplemented with 2.1 M DMSO, 2.7 M ethylene glycol, 0.5 M sucrose and 20%
- 211 FCS. Testis samples were then dropped onto a thin aluminium floater (VWR, Fontenay-sous-
- Bois, France) partially immersed in liquid nitrogen. Vitrified testes were stored in cryotubes
- 213 in liquid nitrogen.
- 214 *Thawing procedure.* Cryotubes were left at room temperature for 2 seconds. Testes were
- incubated in DMEM/F12 supplemented with 0.5 M sucrose and 20 % FCS for 2 minutes at
- 216 37°C, and then in DMEM/F12 supplemented with 20 % FCS for 2 minutes at 37°C. They
- 217 were then successively transferred into four baths of DMEM/F12 supplemented with 20 %
- 218 FCS for 5 minutes at 4° C.
- 219

220 Organotypic cultures

In vitro tissue cultures were performed at a gas-liquid interphase as previously described
(Arkoun et al., 2016; Dumont et al., 2015; Sato et al., 2011; Yokonishi et al., 2014). Briefly,
fresh or frozen/thawed testes were first cut into 4 fragments. They were placed on the top of
two 1.5% (w/v) agarose Type I low EEO gels (Sigma-Aldrich) half-soaked in base medium

225 containing α-MEM, 10% KnockOut Serum Replacement (KSR, Gibco by Life Technologies)

and 5 µg/mL gentamicin (Sigma-Aldrich). Testicular tissues were then cultured in 6-well

227 plates (Dominique Dutscher, Brumath, France) under 5% CO₂ at 34°C for 30 days (D30),

228 which corresponds to the end of the first spermatogenic wave. In the Rol condition, testicular

fragments were cultured with base medium supplemented with 10⁻⁶ M retinol (Sigma-

Aldrich). In the Rol/FSH condition, testicular samples were alternatively cultured with base

231 medium containing 10⁻⁶ M retinol or 500 UI/L recombinant FSH (Gonal-f, Merck Serono,

Lyon, France), with retinol being added every 8 days to coincide with the entry of

spermatogonia into meiosis. In total, 288 testicular fragments from 72 testes were *in vitro*

cultured and analyzed. RT-qPCR analyses were repeated 3 times (from 3 different testes) for

235 *Dnmt1* and *Dnmt3a*, and 3 times for *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1*.

236 Immunohistochemical analyses were repeated 4 times (from 4 different testes) for DNMT1,

237 DNMT3a and 5mC, and 4 times for H3K4me3, H3K9ac and H4K8ac.

238

239 **RNA extraction, reverse transcription and qPCR**

240 **RNA extraction.** Total RNA was extracted from testicular samples (around 2 mg) using

241 RNeasy Micro kit (Qiagen, Courtabœuf, France) according to the manufacturer's instructions.

242 To remove contamination with genomic DNA, RNA was incubated with 2 units of TURBO

243 DNase (Life Technologies) for 45 minutes at 37°C.

244 *Reverse transcription.* cDNA synthesis was performed using qScript cDNA SuperMix

245 (Quanta Biosciences by VWR) according to the manufacturer's instructions using the

following program: 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C.

247 *qPCR.* cDNA amplifications were carried out in a total volume of 20 μ L containing 2 μ L of

cDNA templates diluted 1:10 (10 ng) in sterile water, 10 µL of Fast SYBR Green Master Mix

249 (Applied Biosystems, Saint-Aubin, France) and 300 nM of each primer. Specific primers are

250 listed in Additional File 2. Reactions were performed in 96-well plates (Invitrogen by Life 251 Technologies) in a StepOnePlus thermocycler (Applied Biosystems). Annealing temperatures 252 were optimized for all primer sets (Additional File 2) and the efficiency of the reactions was 253 determined using serial dilutions of cDNA samples. The amplification condition was 20 254 seconds at 95°C followed by 40 cycles (3 seconds at 95°C, 30 seconds at the annealing 255 temperature). Melting curves were also obtained to ensure the specificity of PCR 256 amplifications. The size of the amplicons was verified by agarose gel electrophoresis (E-gel 257 4%, Life Technologies). The relative expression level of each gene (Dnmt1, Dnmt3a, Prdm9, 258 Jarid1b, Src1, Sirt1 and Hdac1) was normalized to the housekeeping gene Gapdh. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. 259

260

261 Histological analyses

262 *Tissue fixation and processing.* Testicular tissues were fixed with Bouin's solution (Sigma-

Aldrich) for 2 hours at room temperature. They were then dehydrated in ethanol in the Citadel

264 2000 tissue processor (Shandon, Cheshire, UK) and embedded in paraffin.

265 *Tissue sectioning.* Tissue sections (3 µm thick) were performed with the JungRM 2035

266 microtome (Leica) and were mounted on Polysine slides (Thermo Fisher Scientific, Waltham,267 MA, USA).

Immunohistochemical staining. Tissue sections were deparaffinized in two baths of xylene
for 5 minutes and rehydrated in decreasing concentrations of ethanol. After a 5-minute wash

- in Tris buffer containing 10% Tween-20 (TBST, Microm Microtech, Francheville, France),
- 271 sections were incubated for 40 minutes at 96° C in 10 mM citrate buffer (pH 6.0) and then
- cooled for 20 minutes at room temperature. Endogenous peroxidases were blocked with HP

273 Block (Dako, Les Ulis, France) for 5 minutes at room temperature. Sections were washed for

5 minutes in distilled water and blocked with Ultra-V blocking solution (Thermo Fisher

275 Scientific) for 10 minutes at room temperature. They were then incubated overnight at 4°C 276 with primary antibodies: rabbit polyclonal anti-DNMT1 IgGs (1:100 for 36-37 dpp in vivo testes and 30-day cultures of testicular tissues, 1:200 for 6-7 dpp testes, ab19905, Abcam, 277 278 Paris, France), rabbit polyclonal anti-DNMT3a IgGs (1:1250, SC-20703, SantaCruz 279 Biotechnology, Heidelberg, Germany), mouse monoclonal anti-5mC IgGs (1:50, 16233D3, 280 Merck Millipore, Fontenay-sous-Bois, France), rabbit polyclonal anti-H3K4me3 IgGs (1:200 281 for 36-37 dpp in vivo testes and 30-day cultures of testicular tissues, 1:500 for 6-7 dpp testes, 282 ab8580, Abcam), rabbit polyclonal anti-H3K9ac IgGs (1:50, ab61231, Abcam) or rabbit 283 polyclonal anti-H4K8ac IgGs (1:1500 for 36-37 dpp in vivo testes and 30-day cultures of 284 testicular tissues, 1:2000 for 6-7 dpp testes, ab15823, Abcam). After a 5-minute wash in 285 TBST, biotinylated goat anti-rabbit antibodies (1:200, Santa Cruz, 25 minutes for DNMT1, 286 DNMT3a and H3K9ac; undiluted, Thermo Fisher Scientific, 5 minutes for H3K4me3 and 287 H4K8ac) or anti-mouse antibodies (Thermo Fisher Scientific, for 5mC) were applied at room 288 temperature. The immunoreaction was visualized after incubation with horseradish 289 peroxidase-conjugated streptavidin (Thermo Fisher Scientific) for 5 minutes at room 290 temperature followed by the addition of 3,3'-diaminobenzidine (Thermo Fischer Scientific) 291 for around 10 seconds (DNMT1 and H3K9ac), 20 seconds (DNMT3a), 30 seconds 292 (H3K4me3) or 40 seconds (5mC and H4K8ac). Sections were finally counterstained with 293 hematoxylin (Dako) for 15 seconds and mounted with Eukitt (CML, Nemours, France). 294 Immunostaining with pre-immune rabbit IgGs (SC-2027, SantaCruz Biotechnology) or mouse 295 IgGs (SC-2025, SantaCruz Biotechnology) was used as a negative control. Images were 296 acquired at a ×500 magnification on a DM4000B light microscope (Leica, Solms, Germany) 297 equipped with the Leica Application Suite Core v2.4 Software. 298 In each testicular sample analyzed, the expression and localization of DNMT1 and DNMT3a, 299 the presence of 5mC residues and modified histones (H3K4me3, H3K9ac and H4K8ac) and

300 the number of each cell type (Sertoli cells: irregular nuclei, spermatogonia: smooth spherical 301 nuclei close to the basal membrane, leptotene/zygotene spermatocytes: irregular spherical 302 nuclei with condensed chromatin, pachytene spermatocytes: irregular spherical nuclei with 303 highly condensed chromatin, round spermatids: small and regular round nuclei, elongated 304 spermatids: elongated nuclei with highly condensed chromatin) per seminiferous tubule were 305 analyzed in 30 cross-sectioned seminiferous tubules from 3 different sections. For in vivo 306 controls, the 30 seminiferous tubules were examined in multiple microscopic fields chosen at 307 random. However, almost all seminiferous tubules present in in vitro matured testes were analyzed because of the small size of the testicular fragments. The percentage of each germ 308 309 cell type that stained positive for DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac 310 was calculated in each seminiferous tubule.

311

312 Sperm DNA methylation

313 *Testicular sperm extraction.* Testicular spermatozoa were extracted from a pool of 6 testes 314 (from 6 different mice) for each condition. Briefly, tissues were placed in α -MEM pre-315 warmed at 37°C. After the careful removal of the central necrotic area using 27-gauge 316 needles, tissues were mechanically dispersed. After a 10-minute centrifugation at 300 × *g*, 317 pellets containing testicular cells were recovered. Cells were fixed with methanol for 30 318 minutes at -20°C and then spread onto glass slides.

319 *Immunofluorescent staining of 5mC*. After 5 minutes in TBST at room temperature, cells

320 were permeabilized in acetone for 2 minutes. Nuclei were decondensed with 1 M NaOH for 4

321 minutes and in TBST containing 10 mM dithiothreitol and 0.1% (v/v) Triton X-100 (Sigma-

322 Aldrich) for 20 minutes at 4°C. Slides were washed twice in TBST for 5 minutes and non-

323 specific binding sites were blocked with 5% (w/v) horse serum albumin in TBST (Sigma-

324 Aldrich). They were then incubated overnight at 4°C with mouse monoclonal anti-5mC IgGs

325 (1:50, 16233D3, Merck Millipore). After two washes in TBST for 5 minutes, Alexa Fluor 488 326 conjugated-goat anti-mouse IgGs (1:200, ab150113, Abcam) were applied for 1 hour at room 327 temperature. After a 10-minute wash, cells were counterstained with 4',6-diamidino-2-328 phenylindole (DAPI, Counterstain I, 30-804931, Adgenix, Voisins-Le-Bretonneux, France). 329 A negative control was carried out by omitting the primary antibody. Images were captured at 330 a ×900 magnification by a U-CMAD3 camera mounted on a BX61 epifluorescence 331 microscope (Olympus), with a fixed acquisition and exposure for each condition. Image 332 acquisitions were carried out using the Bioview 234 Duet v2.3 image analyzer (Nes Ziona, 333 Israël).

334 Fluorescence intensity quantification. For each experimental condition, a hundred 335 spermatozoa (from a pool of 6 different in vitro cultured testes or from 6 in vivo testes), identified by their hook-shaped heads, was examined. The border around the sperm nuclei 336 337 was manually delineated according to DNA staining. After subtracting the background, 5mC 338 and DAPI fluorescence intensities were calculated for each spermatozoon using the ImageJ 339 v1.8 software (National Institute of Health, Bethesda, MD, USA). For each sperm nucleus, 340 the fluorescence of 5mC was divided by the fluorescence of DAPI to obtain normalized 5mC 341 fluorescence.

342

343 Statistical analyses

Statistical analyses were carried out with the statistical GraphPad Prism 6 software (GraphPad
Software Inc., La Jolla, CA, USA). To evaluate the impact of the freezing procedure on
testicular tissue, data obtained from fresh 6-7 *dpp* testes were compared with those obtained
from CSF or SSV 6-7 *dpp* testes using the non-parametric Kruskal Wallis test followed by the
Dunn *post hoc* test. Data from 36-37 *dpp* testes and from 30-day cultured tissues, as well as
data from cultures of fresh, CSF and SSV testicular tissues were also compared using the non-

350 parametric Kruskal Wallis test followed by the Dunn post hoc test. Cultures performed under 351 the Rol or Rol/FSH conditions were compared using the non-parametric Mann Whitney test. 352 Finally, the normalized 5mC fluorescence intensities were compared using the non-parametric 353 Mann Whitney test. Data are expressed as mean \pm SD. For each test, a value of $P \le 0.05$ (*) 354 or P < 0.01 (**) was considered statistically significant. 355 356 Results 357 358 DNA methylation and histone PTMs in fresh or frozen/thawed prepubertal mouse 359 testicular tissue 360 To determine whether the transcripts encoding epigenetic modification enzymes are present in 361 6-7 days *postpartum* (dpp) testes and whether their levels are altered by a freezing/thawing 362 cycle, qPCR analyses were performed on fresh, CSF or SSV testicular tissues. Dnmt1 and 363 Dnmt3a transcripts encoding catalytic DNMTs, Prdm9 and Jarid1b transcripts encoding a 364 histone methyltransferase and demethylase respectively, Src1 transcripts encoding a histone 365 acetyltransferase, and Sirt1 and Hdac1 transcripts encoding histone deacetylases were 366 detected at 6-7 dpp (Figure 1A,B). Moreover, mRNA levels were not significantly different 367 between fresh and frozen/thawed testes, regardless of the freezing/thawing protocol used, 368 except for lower Hdac1 mRNA levels in SSV versus fresh and CSF testes (Figure 1A,B). 369 The expression of the catalytic DNMTs and the presence of the epigenetic marks 5mC, 370 H3K4me3, H3K9ac and H4K8ac were then studied in prepubertal testes by 371 immunohistochemistry (Figure 1C). At 6-7 dpp, seminiferous tubules only contained Sertoli 372 cells and spermatogonia. DNMT1 and 5mC were detected in a majority of spermatogonia, 373 and no significant difference was observed between fresh, CSF and SSV testes (Figure 1D). 374 In contrast, spermatogonia did not express DNMT3a in fresh tissues while around 24% of

375 spermatogonia expressed this enzyme in CSF tissues (P < 0.05, Figure 1D). The three

376 modified histones were found in a minority of spermatogonia in both fresh and frozen/thawed377 testes (Figure 1E).

378

379 Advancement of spermatogenesis in 30-day in vitro cultures of 6-7 dpp testicular tissues 380 As previously described (Arkoun et al., 2016, 2015; Dumont et al., 2017, 2016; Rondanino et 381 al., 2017), a complete spermatogenesis can be achieved *in vitro* within fresh, CSF and SSV 382 prepubertal testicular tissues after 30 days of culture in the presence of 10⁻⁶ M retinol (Rol 383 culture condition). Indeed, germ cells at all stages of differentiation were found after 384 organotypic cultures (Table 1). In an effort to further increase the yield of *in vitro* produced 385 spermatozoa, the supplementation of culture medium with FSH in alternation with retinol 386 (Rol/FSH culture condition) was tested in this study. The progression of spermatogenesis in 387 cultures of fresh, CSF and SSV tissues cultured with Rol or Rol/FSH was evaluated and 388 compared by determining the proportion of the different germ cell types per seminiferous 389 tubule as well as the proportion of seminiferous tubules at the most advanced stage of 390 differentiation. 391 We first compared the progression of spermatogenesis in 30-day cultures of fresh tissues and 392 in the 36-37 dpp age-matched *in vivo* controls. The proportion of pachytene spermatocytes

and of round and elongated spermatids per tubule were lower in cultures of fresh tissues than

in vivo (Table 1). The proportion of tubules having reached the pachytene spermatocyte and

the round spermatid stages was higher in cultures of fresh tissues than in controls (Table 2). In

contrast, the percentage of tubules with elongated spermatids as the most advanced stage was

397 significantly reduced in cultures of fresh tissues compared to *in vivo* (Table 2).

398 The impact of the freezing/thawing procedures on the advancement of spermatogenesis was

then investigated. The different germ cell types were also present in CSF and SSV tissues

400 following in vitro maturation with Rol or Rol/FSH (Table 1). The proportion of pachytene 401 spermatocytes per tubule was similar between cultures of fresh, CSF and SSV tissues in the 402 presence of Rol. With Rol/FSH however, the percentage of pachytene spermatocytes per 403 tubule was higher in cultures of frozen/thawed tissues than in fresh tissues (Table 1). The 404 proportion of round and elongated spermatids per tubule was lower in cultures of CSF tissues 405 than in fresh tissues, whatever the medium used (Table 1). This diminution was not observed 406 in SSV tissues. The proportion of elongated spermatids per seminiferous tubule was even 407 more elevated in cultures of SSV tissues with Rol/FSH than in cultures of CSF tissues (Table 408 1). The percentage of tubules with pachytene spermatocytes as the most advanced type of 409 germ cells was more elevated in CSF tissues matured in the presence of Rol or Rol/FSH and 410 in SSV tissues matured in the presence of Rol/FSH than in fresh tissues (Table 2). The 411 percentage of seminiferous tubules in which round spermatids are the most differentiated 412 germ cells were almost similar in cultures of fresh, CSF and SSV tissues. Indeed, their 413 proportion was only lower in cultures of SSV tissues with Rol/FSH (Table 2). At last, fewer 414 tubules reached the elongated spermatid stage in CSF tissues cultured with Rol or Rol/FSH 415 and in SSV tissues cultured with Rol than in fresh tissues (Table 2). 416 The Rol and Rol/FSH culture conditions were finally compared. The proportion of Sertoli 417 cells, spermatogonia, pachytene spermatocytes and round spermatids per tubule was not 418 significantly different when FSH was added in cultures of fresh or frozen/thawed tissues 419 (Table 1). In addition, the proportion of elongated spermatids per tubule was reduced in 420 cultures of fresh and CSF tissues with Rol/FSH (Table 1). The percentage of tubules in which 421 pachytene spermatocytes are the most differentiated germ cells were more elevated in fresh or 422 SSV tissues cultured with Rol/FSH than with Rol alone (Table 2). Moreover, the percentage 423 of tubules reaching the round spermatid stage was diminished in SSV tissues cultured with 424 Rol/FSH (Table 2). The proportion of tubules containing elongated spermatids was higher

when fresh or CSF tissues were cultured with Rol than with Rol/FSH (Table 2). This
parameter was however not influenced by the medium composition in cultures of SSV tissues.

428 DNA methylation and histone PTMs in 30-day *in vitro* cultures of fresh 6-7 dpp

429 testicular tissues

430 Since the best spermatogenic progression was obtained in tissues cultured with Rol, this

431 culture condition was selected for the rest of the study. The impact of organotypic culture and

432 freezing procedures on the expression of epigenetic modification enzymes, on DNA

433 methylation and histone PTMs was evaluated in *in vitro* matured testicular tissues.

434 Transcript levels were quantified by qPCR in fresh 6-7 dpp testicular tissues cultured *in vitro*

for 30 days and compared with those measured in control 36-37 dpp testes. No significant

436 difference in *Dnmt1* and *Dnmt3a* mRNA levels was found in tissues matured *in vitro* with Rol

437 and *in vivo* controls (Figure 2A). While the levels of *Prdm9* and *Jarid1b* transcripts were

438 similar in cultures of fresh tissues and in controls, *Src1*, *Sirt1* and *Hdac1* mRNA levels were

439 lower *in vitro* (Figure 2B).

440 The expression of the DNMT1 and DNMT3a proteins, DNA methylation onto the C5 position 441 of cytosines and the presence of the three active histone marks were detected in a majority of 442 spermatogonia and leptotene/zygotene spermatocytes in 36-37 dpp testes (Figures 3 and 4). 443 Moreover, more than half of round spermatids contained H3K4me3 and H3K9ac at 36-37 dpp 444 while H4K8ac was found in a lesser proportion of these cells (Figure 4). The percentage of 445 spermatogonia expressing DNMT1 and DNMT3a was similar in fresh tissues cultured with 446 Rol and *in vivo* (Figure 3B). The proportion of leptotene/zygotene spermatocytes expressing 447 DNMT1 in cultures of fresh tissues was also comparable to in vivo controls (Figure 3B). In 448 contrast, a lower percentage of spermatocytes expressing DNMT3a was noticed in tissues 449 cultured with Rol (Figure 3B). The percentage of spermatogonia with methylated DNA was

450 reduced in fresh tissues cultured with Rol compared to 36-37 dpp control testes (Figure 3B). 451 However, the proportion of leptotene/zygotene spermatocytes with 5mC labelling was not 452 significantly different in fresh tissue cultures and in vivo (Figure 3B). DNMT1, DNMT3a and 453 5mC labelling in pachytene spermatocytes, round and elongated spermatids were 454 exceptionally observed in both 36-37 dpp controls and 30-day cultures of 6-7 dpp testicular 455 tissues. A lower proportion of spermatogonia, round and elongated spermatids containing 456 H3K4me3 was found in cultures of fresh tissues than in vivo (P < 0.05, Figure 4B). The 457 percentage of spermatogonia ($P \le 0.05$), round and elongated spermatids ($P \le 0.01$ and $P \le 0.01$) 458 0.05 respectively) containing H3K9ac was also diminished in organotypic cultures (Figure 459 4C). Finally, H4K8ac was found in less spermatogonia and leptotene/zygotene 460 sperrmatocytes in *in vitro* matured fresh tissues (P < 0.05, Figure 4D). 461 462 Epigenetic modifications in 30-day in vitro cultures of CSF or SSV 6-7 dpp testicular 463 tissues 464 To determine whether the freezing and thawing of 6-7 dpp testicular tissues could influence 465 the expression of genes encoding epigenetic modification enzymes in 30-day organotypic 466 cultures, qPCR analyses were performed on in vitro matured CSF or SSV tissues and 467 compared with the data obtained from fresh tissue cultures. Apart from 7.6-fold lower Dnmt1 468 mRNA levels in SSV tissues cultured with Rol (P < 0.05, Figure 2A), Dnmt1 transcript levels 469 did not vary in a significant manner in cultures of CSF tissues with Rol compared to cultures 470 of fresh tissues (Figure 2A). Furthermore, no significant difference in Dnmt3a transcript 471 levels was found after culture of fresh or frozen/thawed testicular tissues (Figure 2A). 472 Moreover, apart from 3.9-fold higher Hdac1 mRNA levels in cultures of SSV tissues, the 473 levels of the transcripts encoding histone modifying enzymes were not significantly different 474 between cultures of fresh, CSF and SSV tissues (Figure 2B). However, lower Dnmt3a and

475 *Hdac1* mRNA levels were found in cultures of CSF tissues and lower *Dnmt1*, *Dnmt3a* and
476 *Jarid1b* mRNA levels were found in cultures of SSV tissues compared to *in vivo* controls
477 (Figure 2).

478 DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac labelling in CSF or SSV tissue 479 cultures were compared to those observed in fresh tissue cultures (Figures 5 and 6). DNMT1, 480 DNMT3a and the 5mC epigenetic mark were found in a majority of spermatogonia in cultures 481 of fresh, CSF or SSV tissues (Figure 5B). Most of the leptotene/zygotene spermatocytes 482 found in cultures of fresh or frozen/thawed tissues expressed DNMT1 and contained 5mC 483 residues (Figure 5B). The proportion of leptotene/zygotene spermatocytes expressing 484 DNMT3a did not appear to be different among those under fresh, CSF, or SSV conditions 485 (Figure 5B). As in the case of fresh tissue cultures, DNMT1, DNMT3a and 5mC were rarely 486 detected in pachytene spermatocytes, round and elongated spermatids. The proportion of 487 elongated spermatids containing H3K4me3 was higher in cultures of CSF and SSV tissues 488 than in fresh tissues (Figure 6B). The proportion of spermatogonia and elongated spermatids 489 containing H3K9ac were more elevated in cultures of CSF tissues than in fresh tissues (Figure 490 6C). This epigenetic mark was also present in a higher proportion of spermatogonia, round 491 and elongated spermatids in cultures of SSV tissues compared to fresh tissues (Figure 6C). 492 Finally, a lower proportion of round spermatids contained H4K8ac in cultures of CSF tissues 493 and the percentage of H4K8ac-postive pachytene spermatocytes was lower in cultures of CSF 494 and SSV tissues than in fresh tissues (Figure 6D).

495

496 DNA methylation in spermatozoa extracted from 30-day *in vitro* cultures of fresh, CSF 497 or SSV 6-7 dpp testicular tissues

498 Despite our attempts using various antigen retrieval procedures, 5mC labelling was rarely
499 detected in germ cells beyond the leptotene/zygotene stage in both *in vivo* controls and *in*

500	vitro matured testicular tissues. In order to ascertain that the genome of <i>in vitro</i> produced
501	gametes was methylated, spermatozoa were extracted from cultures of fresh, CSF or SSV
502	tissues, their nuclei were decondensed and the presence of the heritable 5mC epigenetic mark
503	was investigated by immunofluorescence (Figure 7). The presence of methylated cytosines
504	was detected in the nuclei of spermatozoa. The expression of 5mC between sperm nuclei was
505	heterogeneous. Specifically, the normalized 5mC fluorescence intensity ranged from 0.01 to
506	0.85 (median = 0.16) in spermatozoa extracted from 36-37 d pp testes (Figure 7B). Similar
507	5mC levels were found in spermatozoa produced in cultures of CSF or SSV tissues (median =
508	0.14 and 0.10, respectively), but lower levels were noticed in spermatozoa generated in
509	cultures of fresh tissues (median = 0.10) (Figure 7B). Because of the low spermatogenic yield
510	in cultures, our repeated attempts to analyze the methylation patterns of two imprinted genes
511	(H19 and Igf2r) in extracted spermatozoa by bisulfite pyrosequencing were unsuccessful.
512	

513 **Discussion**

514

515 In the present study, no major alterations of the expression of DNMT1 and DNMT3a, two 516 essential catalytic DNA methyltransferases, and of DNA methylation was found in germ cells 517 after *in vitro* maturation of fresh or frozen/thawed mouse prepubertal testes, when compared 518 to those allowed to develop in vivo. However, differences in the levels of transcripts encoding 519 histone modifying enzymes and in the distribution of the H3K4me3, H3K9ac and H4K8ac 520 marks were found between in vitro and in vivo matured testes. 521 The fertility of childhood cancer survivors could be restored by producing spermatozoa from 522 SSCs with in vivo or in vitro approaches (de Michele et al., 2017; Gies et al., 2015; Ibtisham

523 et al., 2017; Yokonishi and Ogawa, 2016). It has been previously demonstrated that mouse

524 germ cells from fresh prepubertal testes go through typical DNA methylation after SSCs

transplantation and testicular tissue grafting, and that germ cells present alteration in H4K5ac
and H4K8ac marks after SSCs transplantation but not after tissue grafting (Goossens et al.,
2011). If the methylation status of 11 imprinted genes is almost normal in the offspring
derived from *in vitro* produced spermatozoa, the examination of DNA methylation in germ
cells following organotypic culture is warranted, as previously highlighted (Yokonishi et al.,
2014). Moreover, histone PTMs have never been investigated in *in vitro* matured prepubertal
testicular tissues.

532 Fresh or frozen (by CSF or SSV)/thawed 6-7 dpp testicular tissues were first analyzed in this 533 study. Dnmt1 and Dnmt3a gene expression was detected in these testes, which is in 534 accordance with previous data showing that transcripts were present postnatally (La Salle et 535 al., 2004; La Salle and Trasler, 2006; Mertineit et al., 1998). Here, no significant differences 536 in Dnmt1 or Dnmt3a mRNA levels were observed among fresh, CSF and SSV tissues. As 537 previously reported (La Salle et al., 2004; Mertineit et al., 1998), we detected the DNMT1 538 protein in spermatogonia in postnatal testes. In contrast, this enzyme was not found in the 539 mouse prepubertal testis in another study (Goossens et al., 2011). Since the age of the mice 540 was not specified, this discrepancy might come from the analysis of testes at different 541 developmental stages. We observed that spermatogonia present in fresh 6-7 dpp testes 542 contained the 5mC epigenetic mark but did not express DNMT3a. This is consistent with a 543 previous report showing that DNMT3a was only detected in Sertoli and myoid cells while 544 5mC was present in spermatogonia (Goossens et al., 2011). The presence of methylated cytosines in spermatogonia indicates that methylation profiles were, at least in part, 545 546 established at this stage. Indeed, following a demethylation wave during embryonic 547 development, spermatogonial precursor cells and spermatogonia then undergo de novo DNA 548 methylation (Boissonnas et al., 2013; Kubo et al., 2015; Yao et al., 2015). While the presence 549 of DNMT1 suggests that DNA methylation profiles are maintained during SSC division, the

550 lack of DNMT3a may however mean that *de novo* methylation is not occurring in 551 spermatogonia at 6-7 dpp. In the present study, DNMT1 expression and 5mC marks were also 552 observed in spermatogonia of thawed CSF or SSV testes. For unknown reasons and contrary 553 to fresh tissues, DNMT3a was found in spermatogonia in frozen/thawed tissues, and may 554 cause in these cells the appearance of abnormal DNA methylation profiles and the repression 555 of gene expression. Moreover, we showed that the *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1* 556 genes encoding histone modifying enzymes were expressed in fresh, CSF and SSV 6-7 dpp 557 testes. In contrast to a previous study (Goossens et al., 2011), we detected the H3K9ac mark 558 not only in Sertoli cells but also in a small fraction of spermatogonia. The H3K4me3 and 559 H4K8ac marks were also present in these two cell types, as previously described (Goossens et 560 al., 2011).

561 Before analyzing DNA methylation and histone PTMs in *in vitro* matured prepubertal 562 testicular tissues, the advancement of spermatogenesis was examined. As previously shown 563 (Arkoun et al., 2016, 2015; Dumont et al., 2016, 2015; Sato et al., 2011; Yokonishi et al., 564 2014), a successful differentiation of spermatogonia into spermatozoa was obtained in the 565 present study after culture of fresh or frozen/thawed prepubertal testicular tissues at a gas-566 liquid interphase. The proportion of elongated spermatids per seminiferous tubule and of 567 tubules having reached the elongated spermatid stage were lower in 30-day cultures of fresh 568 tissues compared to *in vivo* controls. Moreover, these parameters were reduced in CSF tissues 569 compared to fresh or SSV tissues. Our data therefore suggest that vitrification better preserves 570 testicular tissues than slow freezing, and are in line with our previous data reporting better 571 spermatogenic yields in cultures of SSV than CSF tissues (Dumont et al., 2015). To promote 572 germ cell differentiation, tissues were cultured with retinol alone or with an alternation 573 retinol/FSH. Indeed, probably because of the complex interactions between retinoids and FSH 574 (Livera et al., 2002), no additive effect has been previously observed on in vitro

575 spermatogenesis when retinol and FSH were combined together (Arkoun et al., 2015). We 576 found that the proportion of elongated spermatids per tubule and of tubules containing 577 elongated spermatids was significantly reduced in fresh and CSF tissues cultured with 578 retinol/FSH rather than retinol alone. In order to mimic in vivo conditions as closely as 579 possible, different concentrations of FSH (corresponding to the physiological variations of 580 this hormone) will have to be used during the course of *in vitro* cultures in future studies. 581 Moreover, the culture medium will have to be supplemented with other factors that positively 582 influence spermatogenesis. Indeed, recently, the use of a chemically-defined medium 583 containing retinoic acid, retinol and a combination of four hormones (FSH, LH, testosterone, 584 triiodothyronine) was found to promote spermatogenesis until round spermatid production 585 (Sanjo et al., 2018).

In this study, *Dnmt1* and *Dnmt3a* transcripts were detected *in vivo* at 36-37 dpp and in fresh,

587 CSF and SSV tissues cultured for 30 days. Differences in their levels were evidenced between 588 in vivo controls and cultures of fresh or frozen/thawed tissues. DNMT1, DNMT3a and 5mC 589 residues were mostly localized in spermatogonia and leptotene/zygotene spermatocytes in 590 vivo and in cultures of fresh or frozen/thawed tissues. The presence of DNMT1 and DNMT3a 591 specifically in spermatogonia and leptotene/zygotene spermatocytes during spermatogenesis 592 is in agreement with previous studies (La Salle and Trasler, 2006; Mertineit et al., 1998; 593 Watanabe et al., 2004). In adult testis, DNMT1 has also been detected in pachytene 594 spermatocytes, diplotene spermatocytes, round spermatids and 5mC was also found in round spermatids (Goossens et al., 2011). The expression of DNMT3a in 36-37 dpp testes and 30-595 596 day cultures suggests that *de novo* methylation is occurring at this stage. Here, the epigenetic 597 5mC mark was not found in germ cells beyond the leptotene/zygotene spermatocyte stage in 598 vivo and in organotypic cultures. As suggested previously (Goossens et al., 2011), the lack of 599 5mC staining in pachytene spermatocytes could be due to the fact that the methylation pattern

600 has not yet been set on daughter DNA strand. The lack of labelling in spermatids may 601 however be explained by a poor accessibility of antibodies to the condensed DNA. To 602 ascertain that DNA methylation marks are indeed found in most differentiated germ cells, we 603 extracted spermatozoa from the testicular tissues after culture and performed 5mC labelling 604 on decondensed DNA. The epigenetic mark 5mC was detected in the nuclei of spermatozoa 605 generated in cultures of fresh or frozen/thawed testicular tissues. The relative 5mC 606 fluorescence intensity was similar in spermatozoa produced in cultures of frozen/thawed 607 tissues or *in vivo*, but was lower in spermatozoa extracted from cultures of fresh tissues. It has been shown that the DNA methylation patterns of a paternally methylated gene (Igf2) and a 608 609 maternally expressed gene (*Peg1*) in epididymal spermatozoa obtained after SSC 610 transplantation were not different from controls (Goossens et al., 2009). In our study, the 611 analysis of the methylation status of the paternally imprinted gene H19 and the maternally 612 imprinted gene Igf2r in in vitro produced spermatozoa could not be achieved. Indeed, a 613 heterogeneous cell population, among which few spermatozoa, was obtained after mechanical 614 dissociation of in vitro matured testes. 615 In addition, our data showed that Prdm9, Jarid1b, Src1, Sirt1 and Hdac1 transcripts were also 616 present in 36-37 dpp testes and in fresh or frozen/thawed tissues cultured for 30 days. The 617 levels of Src1, Sirt1 and Hdac1 mRNA encoding histone acetyltransferase or deacetylase 618 were lower in cultures of fresh tissues than *in vivo*. However, apart from lower mRNA levels 619 of Jarid1b in cultures of SSV tissues and of Hdac1 in cultures of CSF tissues, the levels of 620 transcripts encoding the different histone modifying enzymes were similar between cultures 621 of frozen/thawed tissues and in vivo. As previously reported (Godmann et al., 2007; Goossens 622 et al., 2011; Hazzouri et al., 2000; Zhang et al., 2014), we found that H3K4me3, H3K9ac and 623 H4K8ac were present in spermatogonia, leptotene/zygotene spermatocytes, round and 624 elongated spermatids and in few pachytene spermatocytes in vivo. An altered distribution of

625 these three active histone marks was observed in our organotypic cultures. We observed that 626 the H3K4me3 and H3K9ac marks, which are involved in the progression of early meiosis and 627 spermiogenesis respectively (Hayashi et al., 2005; Bell et al., 2014), were present in a lesser 628 proportion of spermatogonia, round and elongated spermatids and in a higher proportion of 629 pachytene spermatocytes in cultures of fresh tissues compared to in vivo controls. It has been 630 shown that a decrease in H3K4me3 or in H3K9ac could impair meiotic progression or induce 631 morphological alterations and apoptosis in round spermatids respectively (Hayashi et al., 632 2005; Dai et al., 2015). This could explain, at least in part, the alteration of meiotic and 633 postmeiotic progression and the presence of degenerating round spermatids in in vitro 634 matured tissues as well as the low spermatogenic yield previously reported by us and others 635 (Dumont et al., 2016; Rondanino et al., 2017; Yao et al., 2017). The acetylation of histones, 636 which precedes histone-to-protamine replacement, was probably unaffected in the surviving 637 spermatids since most of *in vitro* produced spermatozoa contain normally condensed 638 chromatin (Oblette et al., 2017). Finally, while H4K8ac was found in less spermatogonia and 639 leptotene/zygotene spermatocytes in our cultures of fresh tissues, the proportion of spermatids 640 containing this mark was similar to in vivo controls. The freezing/thawing procedures used in 641 this study also altered the distribution of the histone marks. A disturbed expression of histone 642 modifying enzymes (PRDM9, JARID1B, SRC1, SIRT1, HDAC1 or others) or of their 643 activity in organotypic cultures could explain these results. 644 Genome-wide single-cell methylome analysis and chromatin immunoprecipitation sequencing 645 will be necessary to explore in more detail DNA methylation in in vitro produced 646 spermatozoa, and especially the methylation status of imprinted genes, as well as the genomic 647 distribution of modified histones.

648

649 In conclusion, we here report for the first time that germ cells go through DNA methylation 650 and histone PTMs when in vitro matured. Importantly, in the context of fertility preservation, 651 the present study was also performed on frozen/thawed samples. DNA methylation was found 652 in cultures of frozen/thawed prepubertal testicular tissues and in spermatozoa generated in 653 these tissues. Histone methylation and acetylation also occurred in germ cells in cultures of 654 cryopreserved tissues. Before applying *in vitro* maturation to frozen/thawed prepubertal 655 testicular tissues of pediatric cancer survivors, further studies will be necessary to investigate 656 whether imprinted genes are properly methylated in *in vitro* produced spermatozoa, whether 657 histone marks localize to the correct regions of the genome, and whether first and second-658 generation offspring are healthy, long-living and do not present behavioral disorders.

659

660 Vitae

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665

666 List of abbreviations

667 CSF: controlled slow freezing; DNMT: DNA methyltransferase; dpp: days postpartum; FSH:

668 follicle stimulating hormone; H3K4me3: trimethylated histone H3 on lysine 4; H3K9ac:

acetylated histone H3 on lysine 9; H4K8ac: acetylated histone H4 on lysine 8; KSR:

670 KnockOut serum replacement; 5mC: 5-methylcytosine; PTMs: post-translational

671 modifications; Rol: retinol; SSC: spermatogonial stem cells; SSV: solid surface vitrification

672 Acknowledgements. The authors are grateful to Véronique Duchesne, Amandine Bironneau

and Shirley Troche for excellent technical assistance.

674 **Competing interests.** The authors declare that they have no competing interests.

675 *Authors' contributions.* AO performed experiments, analyzed data and wrote the manuscript;676 JR performed experiments, analyzed data and revised the manuscript; LD, MD, JS and AR677 analyzed data and revised the manuscript; NR supervised the study and revised the678 manuscript; CR designed and supervised the study and wrote the manuscript. All the authors679 approved the final version of the manuscript.

680

681 **Funding.** This work was supported by Rouen University Hospital, Ligue contre le Cancer [to

NR and AO], Agence de la Biomédecine [to NR], Association Laurette Fugain [to NR and

683 CR], France Lymphome Espoir [to NR], and co-supported by European Union and Région

684 Normandie [to AO]. Europe gets involved in Normandie with European Régional

685 Development Fund (ERDF). The funding sources had no role in the design of the study,

686 collection, analysis and interpretation of data, and in writing the manuscript.

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- 914
- 915
- 916 Figure legends

9

- 917
- 918 Figure 1. Expression of epigenetic modification enzymes, DNA methylation and histone

919 post-translational modifications in fresh and frozen/thawed prepubertal mouse testes.

920 (A) Relative mRNA levels of the *Dnmt1* and *Dnmt3a* genes normalized to the housekeeping

gene *Gapdh* in fresh, CSF and SSV 6-7 dpp testes (n=3). Histograms are expressed as mean ±
SD. *P < 0.05.

923 (B) Relative mRNA levels of the *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1* genes normalized to

the housekeeping gene *Gapdh* in fresh, CSF and SSV 6-7 dpp testes (n=3). Histograms are

925 expressed as mean \pm SD. **P* < 0.05.

926 (C) Localization of DNMT1, DNMT3a and the 5mC, H3K4me3, H3K9ac and H4K8ac

927 epigenetic marks in fresh, CSF and SSV 6-7 dpp testes (n=4). Shown are representative

928 microscopy images of DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac labelling.

- 929 Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and tissue
- 930 sections were counterstained with hematoxylin. Enlarged views of labelled and unlabeled
- 931 spermatogonia are shown in the upper right corner and in the lower right corner, respectively.
- 932 Bar: 50 μm.

- 933 (**D**) Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia in 30 cross-sectioned 934 seminiferous tubules of fresh, CSF and SSV 6-7 d*pp* testes (n=4). Histograms are expressed 935 as mean \pm SD. **P* < 0.05.
- 936 (E) Proportion of H3K4me3, H3K9ac and H4K8ac-positive spermatogonia in 30 cross-
- 937 sectioned seminiferous tubules of fresh, CSF and SSV 6-7 dpp testes (n=4). Histograms are
- 938 expressed as mean \pm SD. **P* < 0.05.
- 939 CSF: controlled slow freezing; *Dnmt*/DNMT: DNA methyltransferase; *dpp*: days *postpartum*;
- 940 *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; H3K4me3: trimethylated histone H3 on
- 941 lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated histone H4 on
- 942 lysine 8; *Hdac1*: Histone deacetylase 1; *Jarid1b*: Jumonji AT-rich interactive domain 1b;
- 943 5mC: 5-methylcytosine; *Prdm9*: PR domain-containing 9; *Sirt1*: Sirtuin 1; *Src1*: Steroid
- 944 receptor coactivator 1; SSV: solid surface vitrification
- 945
- **Figure 2.** Expression of genes encoding epigenetic modification enzymes in cultured
- 947 testicular tissues and *in vivo* controls.
- 948 Relative mRNA levels of the (A) *Dnmt* genes (*Dnmt1*, *Dnmt3a*) and (B) genes encoding
- histone modifying enzymes (*Prdm9*, *Jarid1b*, *Src1*, *Sirt1*, *Hdac1*) normalized to the
- 950 housekeeping gene Gapdh in 30-day cultures of fresh, CSF or SSV testicular fragments with
- retinol (n = 3) and in testes of mice aged 36–37 dpp (n = 3). Histograms are expressed as
- 952 mean \pm SD. **P* < 0.05, ***P* < 0.01.
- 953 CSF: controlled slow freezing; D30: day 30; *Dnmt*: DNA methyltransferase; dpp: days
- 954 *postpartum*; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; *Hdac1*: Histone
- 955 deacetylase 1; Jarid1b: Jumonji AT-rich interactive domain 1b; Prdm9: PR domain-
- 956 containing 9; Rol: retinol; *Sirt1*: Sirtuin 1; *Src1*: Steroid receptor coactivator 1; SSV: solid
- 957 surface vitrification

959	Figure 3. Immunolocalization of DNMT1, DNMT3a and 5mC in cultures of fresh
960	prepubertal testes and in vivo.
961	(A) Localization of DNMT1, DNMT3a and the 5mC epigenetic mark in fresh testicular
962	fragments cultured for 30 days with retinol (n=4) and in 36-37 dpp testes (in vivo controls,
963	n=4). Shown are representative microscopy images of DNMT1, DNMT3a and 5mC labelling.
964	Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and tissue
965	sections were counterstained with hematoxylin. Enlarged views of spermatogonia and
966	leptotene/zygotene spermatocytes are shown in the upper right corner and in the lower right
967	corner, respectively. Bar: 50 μm.
968	(B) Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia and
969	leptotene/zygotene spermatocytes in 30-day cultures of fresh testicular fragments with retinol
970	(n=4) and in control 36-37 dpp testes (n=4). The localization of DNMT1, DNMT3a and 5mC
971	was analyzed in 30 cross-sectioned seminiferous tubules from three sections. Histograms are
972	expressed as mean \pm SD. * <i>P</i> < 0.05.
973	D30: day 30; DNMT: DNA methyltransferase; dpp: days postpartum; 5mC: 5-
974	methylcytosine; L/Z: leptotene/zygotene spermatocyte; P: pachytene spermatocyte; Rol:
975	retinol; Sg: spermatogonia
976	
977	Figure 4. Immunolocalization of H3K4me3, H3K9ac and H4K8ac in cultures of fresh
978	prepubertal testes and in vivo.
979	(A) Localization of the epigenetic marks H3K4me3, H3K9ac and H4K8ac in fresh testicular
980	fragments cultured for 30 days with retinol (n=4) and in 36-37 dpp testes (in vivo controls,
981	n=4). Shown are representative microscopy images of H3K4me3, H3K9ac and H4K8ac
982	labelling. Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and

- 983 tissue sections were counterstained with hematoxylin. Enlarged views of spermatogonia,
- 984 leptotene/zygotene and pachytene spermatocytes (upper right corner) and of round and

985 elongated spermatids (lower right corner) are shown. Bar: 50 μm.

- 986 (**B-D**) Proportion of (**B**) H3K4me3, (**C**) H3K9ac and (**D**) H4K8ac-positive spermatogonia,
- 987 leptotene/zygotene and pachytene spermatocytes, round and elongated spermatids in 30-day
- 988 cultures of fresh testicular fragments with retinol (n=4) and in control 36-37 dpp testes (n=4).
- 989 The localization of H3K4me3, H3K9ac and H4K8ac was analyzed in 30 cross-sectioned
- 990 seminiferous tubules from three sections. Histograms are expressed as mean \pm SD. **P* < 0.05,
- 991 **P < 0.01.
- 992 D30: day 30; dpp: days postpartum; ES: elongated spermatid; H3K4me3: trimethylated
- histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated
- histone H4 on lysine 8; L/Z: leptotene/zygotene spermatocyte; P: pachytene spermatocyte;

895 Rol: retinol; RS: round spermatid; Sg: spermatogonia

- 996
- Figure 5. Immunolocalization of DNMT1, DNMT3a and 5mC in 30-day cultures offrozen/thawed prepubertal testes.
- 999 (A) Localization of DNMT1, DNMT3a and 5mC in CSF or SSV testicular fragments cultured
- 1000 for 30 days with retinol (n=4). Shown are representative microscopy images of DNMT1,
- 1001 DNMT3a and 5mC labelling. Antibody binding was visualized using 3,3'-diaminobenzidine
- 1002 (brown staining) and tissue sections were counterstained with hematoxylin. Enlarged views of
- 1003 spermatogonia and leptotene/zygotene spermatocytes are shown in the upper right corner and
- 1004 in the lower right corner, respectively. Bar: 50 μm.
- 1005 (B) Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia and
- 1006 leptotene/zygotene spermatocytes in CSF or SSV testicular fragments cultured for 30 days
- 1007 with retinol (n=4). The localization of DNMT1, DNMT3a and 5mC was analyzed in 30 cross-

1008 sectioned seminiferous tubules from three sections. Histograms are expressed as mean \pm SD. 1009 **P* < 0.05.

1010 CSF: controlled slow freezing; D30: day 30; DNMT: DNA methyltransferase; dpp: days

1011 *postpartum*; 5mC: 5-methylcytosine; L/Z: leptotene/zygotene spermatocyte; P: pachytene

1012 spermatocyte; Rol: retinol; Sg: spermatogonia; SSV: solid surface vitrification

1013

1014 Figure 6. Immunolocalization of H3K4me3, H3K9ac and H4K8ac in cultures of1015 frozen/thawed prepubertal testes.

1016 (A) Localization of H3K4me3, H3K9ac and H4K8ac in CSF or SSV testicular fragments

1017 cultured for 30 days with retinol (n=4). Shown are representative microscopy images of

1018 H3K4me3, H3K9ac and H4K8ac labelling. Antibody binding was visualized using 3,3'-

1019 diaminobenzidine (brown staining) and tissue sections were counterstained with hematoxylin.

1020 Enlarged views of spermatogonia, leptotene/zygotene and pachytene spermatocytes (upper

right corner) and of round and elongated spermatids (lower right corner) are shown. Bar: 50

1022 μm.

1023 (**B-D**) Proportion of (**B**) H3K4me3, (**C**) H3K9ac and (**D**) H4K8ac-positive spermatogonia,

1024 leptotene/zygotene and pachytene spermatocytes, round and elongated spermatids in CSF and

1025 SSV fragments cultured for 30 days with retinol (n=4). The localization of H3K4me3,

1026 H3K9ac and H4K8ac was analyzed in 30 cross-sectioned seminiferous tubules from three

1027 sections. Histograms are expressed as mean \pm SD. **P* < 0.05.

1028 D30: day 30; dpp: days postpartum; ES: elongated spermatid; H3K4me3: trimethylated

1029 histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated

1030 histone H4 on lysine 8; L/Z: leptotene/zygotene; P: pachytene spermatocyte; Rol: retinol; RS:

1031 round spermatid; Sg: spermatogonia

1032

1033 Figure 7. DNA methylation in *in vitro* produced spermatozoa and *in vivo* controls.

1034 (A) 5mC patterns in spermatozoa extracted from fresh, CSF or SSV testes after 30 days of

1035 culture in the presence of retinol, and from 36-37 dpp testes (*in vivo* controls). A hundred

- 1036 spermatozoa (from a pool of 6 *in vitro* matured testes or from 6 *in vivo* testes) were examined.
- 1037 Shown are representative images of 5mC immunofluorescence staining (in green) in
- 1038 spermatozoa. Cell nuclei were stained with DAPI (in blue). Representative merged
- 1039 fluorescent images obtained when the primary antibody was omitted (negative controls) are
- 1040 also shown (right column). Magnification: \times 900, bar: 10 μ m.
- 1041 (B) Box plot distributions of normalized 5mC fluorescence intensity in spermatozoa extracted

1042 from fresh, CSF or SSV testes after 30 days of culture in the presence of retinol, and from 36-

1043 37 dpp testes (*in vivo* controls). The boxes stretch from the 25^{th} to the 75^{th} percentile. The

- 1044 median is shown as a line across the boxes. The horizontal lines outside the boxes display 1045 minimum and maximum. The circles represent outliers. **P < 0.01.
- 1046 CSF: controlled slow freezing; D30: day 30; DAPI: 4',6-diamidino-2-phenylindole; dpp: day
- 1047 *postpartum*; 5mC: 5-methylcytosine; Rol: retinol; SSV: solid surface vitrification
- 1048

1049 Additional File 1. Schematic representation of the experimental design

1050 Testes from 6-7 dpp prepubertal CD-1 mice were obtained and organotypic cultures were

- 1051 performed either directly (from fresh tissues) or after thawing of testes frozen by CSF or SSV.
- 1052 Testicular fragments were cultured at a gas-liquid interphase on agarose gels (under 5% CO₂
- 1053 at 34°C), with retinol alone or a retinol/FSH alternation ("Rol" and "Rol/FSH" culture
- 1054 conditions, respectively) for 30 days. Testes from mice aged 36-37 dpp represent the
- 1055 corresponding *in vivo* controls. After selection of the best culture condition, *in vitro* matured
- 1056 testicular tissues and *in vivo* controls were analyzed by RT-qPCR and by
- 1057 immunohistochemistry. Moreover, a hundred spermatozoa extracted from a pool of 6 in vitro

- 1058 cultured testes or from 6 *in vivo* testes were analyzed by immunofluorescence. In order to
- 1059 evaluate the impact of the freezing procedures on the testicular tissues before culture, fresh,
- 1060 CSF and SSV testes from mice aged 6-7 dpp were also analyzed by RT-qPCR and
- 1061 immunohistochemistry. A total of 134 testes from 73 mice were used in this study. RT-qPCR
- analyses were repeated 3 times (from 3 different testes) and immunohistochemical analyses
- 1063 were repeated 4 times (from 4 different testes). For the analysis of sperm DNA methylation, a
- 1064 pool of 6 testes (from 6 different mice) was used to extract spermatozoa.
- 1065 (*) The same testicular samples were used for the evaluation of spermatogenic progression
- 1066 and for DNMT1, DNMT3a, 5mC immunostaining
- 1067 α-MEM: minimum essential medium α; CSF: controlled slow freezing; *dpp*: days *postpartum*;
- 1068 *Dnmt*/DNMT: DNA methyltransferase; FSH: follicle stimulating hormone; H3K4me3:
- trimethylated histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac:
- 1070 acetylated histone H4 on lysine 8; *Hdac1*: Histone deacetylase 1; *Jarid1b*: Jumonji AT-rich
- 1071 interactive domain 1b; KSR: *knockout* replacement serum; 5mC: 5-methylcytosine; *Prdm9*:
- 1072 PR domain-containing 9; Rol: retinol; RT-qPCR: quantitative PCR after reverse transcription;
- 1073 Sirt1: Sirtuin 1; Src1: Steroid receptor coactivator 1; SSV: solid surface vitrification



Figure 2



В



В

Unlabelled Labelled

DNMT3a







5mC





В



L/Z







В



	% of cell types present in seminiferous tubules sections						
	36-37 d <i>pp</i>	Fresh + Rol D30	Fresh + Rol/FSH D30	CSF + Rol D30	CSF + Rol/FSH D30	SSV + Rol D30	SSV + Rol/FSH D30
Sertoli cells	9.11	33.13	29.64	39.17	37.33	33.59	29.38
	(2.43)	(2.50) ^a	(5.96) ^a	(4.44) ^b	(4.78) ^c	(6.14) ^d	(5.90) ^e
Spermatogonia	7.89	14.78	14.45	20.12	19.18	15.94	15.65
	(1.53)	(4.61) ^a	(3.31) ^a	(4.49) ^b	(1.92) ^c	(4.04)	(4.49) ^e
Leptotene/zygotene	3.24	4.91	11.91	3.20	9.28	3.34	5.69
spermatocytes	(1.64)	(5.39)	(5.63) ^{a,b}	(1.53)	(4.28) ^d	(1.85)	(4.50) ^f
Pachytene	24.40	22.50	21.63	26.85	27.06	27.42	32.90
spermatocytes	(2.97)	(6.12) ^a	(3.86) ^a	(5.60)	(6.25) ^c	(4.64)	(4.27) ^c
Round spermatids	37.70	15.15	15.13	7.31	5.18	13.83	11.83
	(2.55)	(1.95) ^a	(5.34) ^a	(3.75) ^b	(2.60) ^c	(4.75) ^d	(3.70) ^e
Elongated spermatids	17.66	9.53	7.24	3.35	1.97	5.88	4.55
	(4.32)	(3.06) ^a	(3.27) ^{a.b}	(1.05) ^b	(1.50) ^{c.d}	(2.20)	(2.53) ^e

 Table 1. Assessment of germ cell differentiation in seminiferous tubules

Data are presented as the mean (SD) with n = 4 for 36-37 dpp, Fresh + Rol D30, Fresh +

Rol/FSH D30, CSF + Rol D30, CSF + Rol/FSH D30, SSV + Rol D30, SSV + Rol/FSH D30.

CSF, controlled slow freezing; D30, 30-day tissue culture; dpp, days postpartum; Rol, retinol;

Rol/FSH, retinol/follicle stimulating hormone; SSV, solid surface vitrification.

^aValues significantly different with 36-37 dpp (P < 0.05)

^bValues significantly different with Fresh + Rol D30 ($P \le 0.05$)

^cValues significantly different with Fresh + Rol/FSH D30 (P < 0.05)

^dValues significantly different with CSF + Rol D30 ($P \le 0.05$)

^eValues significantly different with CSF + Rol/FSH D30 ($P \le 0.05$)

^fValues significantly different with SSV + Rol D30 (P < 0.05)

Table 2. Percentage of seminiferous tubules containing differentiated germ cells at the most

 advanced stage

	% of seminiferous tubules at the most advanced stage						
	36-37 d <i>pp</i>	Fresh + Rol D30	Fresh + Rol/FSH D30	CSF + Rol D30	CSF + Rol/FSF D30	SSV I+Rol D30	SSV + Rol/FSH D30
Spermatogonia	0	0.28 (0.96)	2.05 (2.72) ^a	3.15 (5.90)	6.17 (8.03)	3.33 (5.50)	6.13 (7.85)
Leptotene/zygotene spermatocytes	0	0.28 (0.96)	5.13 (5.89) ^{a.b}	0	6.94 (6.12) ^d	1.94 (4.13)	2.73 (2.29)
Pachytene spermatocytes	0	16.75 (5.08) ^a	27.64 (8.59) ^{a.b}	50.88 (10.82) ^b	55.37 (11.87) ^c	30.56 (15.10) ^d	47.11 (15.46) ^{c.e}
Round spermatids	4.72 (3.76)	33.45 (11.76) ^a	29.07 (9.46) ^a	23.33 (9.64)	18.12 (10.61)	31.11 (9.57)	17.67 (3.43) ^{c.e}
Elongated spermatids	95.28 (3.61)	49.24 (10.86) ^a	36.11 (10.76) ^{a.b}	22.64 (5.92) ^b	13.40 (7.60) ^{c.d}	33.06 (11.41) ^b	26.36 (14.89)

Data are presented as the mean (SD) with n = 4 for 36-37 dpp, Fresh + Rol D30, Fresh +

Rol/FSH D30, CSF + Rol D30, CSF + Rol/FSH D30, SSV + Rol D30, SSV + Rol/FSH D30.

CSF, controlled slow freezing; D30, 30-day tissue culture; dpp, days postpartum; Rol, retinol;

Rol/FSH, retinol/follicle stimulating hormone; SSV, solid surface vitrification.

^aValues significantly different with 36-37 dpp (P < 0.05)

^bValues significantly different with Fresh + Rol D30 (P < 0.05)

^cValues significantly different with Fresh + Rol/FSH D30 ($P \le 0.05$)

^dValues significantly different with CSF + Rol D30 ($P \le 0.05$)

^eValues significantly different with SSV + Rol D30 ($P \le 0.05$)