THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Irinotecan metabolite SN38 results in germ cell loss in the testis but not in the ovary of prepubertal mice

OPEN ACCESS

1   **Irinotecan metabolite SN38 results in germ cell loss in the testis but not in the ovary of**

2   **prepubertal mice**

3

4   **Authors**

5

6   Federica Lopes[1], Rowena Smith[1], Sophie Nash[1], Rod T. Mitchell[2], Norah Spears[1]*

7

8   **Addresses**

9

10   [1] Centre for Integrative Physiology, University of Edinburgh, Edinburgh, EH8 9XD, United Kingdom [2]

11   MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, EH16 4TJ, United Kingdom

12

13   **Corresponding author**

14

15   * E-mail: norah.spears@ed.ac.uk

16

17   **Running title:** SN38 depletes germ cells only in male mice

18

19

20

21

22

28 **Abstract**

29 **Study question:** Does the Irinotecan metabolite 7-ethyl-10-hydroxycamptothecan (SN38) damage
30 the gonads of male and female prepubertal mice?

31

32 **Summary answer:** The Irinotecan metabolite SN38 reduces germ cell numbers within the
33 seminiferous tubules of mouse testes at concentrations that are relevant to cancer patients, while in
34 contrast it has little if any effect on the female germ cell population.

35

36 **What is known already:** Little is known about the role of the chemotherapeutic agent Irinotecan on
37 female fertility, with only one article to date reporting menopausal symptoms in perimenopausal
38 women treated with Irinotecan, while no data are available either on adult male fertility or on the
39 impact of Irinotecan on the subsequent fertility of prepubertal cancer patients, female or male.

40

41 **Study design, size, duration:** Male and female gonads were obtained from postnatal day 5 C57BL/6
42 mice and exposed in vitro to a range of concentrations of the Irinotecan metabolite SN38: 0.002,
43 0.01, 0.05, 0.1 or 1 μg ml^{-1} for the testis and 0.1, 1, 2.5 or 5 μg ml^{-1} for the ovary, with treated gonads
44 compared to control gonads not exposed to SN38: SN38 was dissolved in 0.5% dimethyl sulfoxide,
45 with controls exposed to the same concentration of diluent. The number of testis fragments used for
46 each analysis ranged between 3 and 9 per treatment group, while the number of ovaries used for
47 each analysis ranged between 4 and 12 per treatment group.

48 **Participants/materials, setting, methods:** Neonatal mouse gonads were developed in vitro, with
49 tissue analysed at the end of the 4-6 day culture period, following immunofluorescence or
50 hematoxylin and eosin staining. Statistical analyses were performed using one-way ANOVA followed
51 by Bonferroni post-hoc test for normally distributed data and Kruskal-Wallis test followed by Dunns
52 post-test for non-parametric data.

53 **Main results and the role of chance:** Abnormal testis morphology was observed when tissues were
54 exposed to SN38, with a smaller seminiferous tubule diameter at the highest concentration of SN38
55 (1 μg ml^{-1}, $p<0.001$ versus control) and increased number of Sertoli cell-only tubules at the two
56 highest concentrations of SN38 (0.1 μg ml^{-1}, $p<0.001$; 1 μg ml^{-1}, $p<0.0001$, both versus control).
57 Within seminiferous tubules, a dose response decrease was observed in both germ cell number
58 (determined by the number of mouse vasa homologue (MVH)-positive cells) and in proliferating cell
59 number (determined by the number of bromodeoxyuridine (BrdU)-positive cells), with significance
60 reached at the two highest concentrations of SN38 (0.1 μg ml^{-1}, $p<0.01$ for both; 1 μg ml^{-1}, $p<0.001$-
61 MVH, $p<0.01$-BrdU; all versus control). No change was seen in protein expression of the apoptotic
62 marker cleaved caspase 3. Double immunofluorescence showed that occasional proliferating germ
63 cells were present in treated testes, even after exposure to the highest drug concentration. When
64 prepubertal ovaries were treated with SN38, no effect was seen on germ cell number, apoptosis, or
65 cell proliferation, even after exposure to the highest drug concentrations.

66 **Limitations, reasons for caution:** As with any study using in vitro experiments with an experimental
67 animal model, caution is required when extrapolating the present findings to humans. Differences
68 between human and mouse spermatogonial development also need to be considered when
69 assessing the effect of chemotherapeutic exposure. However, the prepubertal testes and ovaries
70 used in the present studies contain germ cell populations that are representative of those found in

2

71 prepubertal patients, and experimental tissues were exposed to drug concentrations within the

72 range found in patient plasma.

73 **Wider implications of the findings:** Our findings demonstrate that the prepubertal mouse ovary is

74 relatively insensitive to exposure to the Irinotecan metabolite SN38, while it induces a marked dose-

75 dependent sensitivity in the testicular germ cell population. The study identifies the importance of

76 further investigation to identify the risk of infertility in young male cancer patients treated with

77 Irinotecan.

78 **Large scale data:** n/a

82

83 **Key words:** chemotherapy, fertility preservation, testis, spermatogonia, ovary, oocyte,

84 gonadotoxicity, tissue culture, apoptosis, cell proliferation

85

Introduction

Recent advances in cancer treatment have significantly increased life expectancy, especially for young patients. Since most of these oncological therapies do not have cancer cell-specific action, they can also affect healthy cells, impairing important physiological processes. One of the major concerns for younger cancer patients is the risk of infertility as a result of treatment (Anderson *et al.* , 2015). Although not every chemotherapeutic drug impairs fertility, some (e.g. alkylating agents) are recognised to be particularly gonadotoxic (Meistrich, 2013, Meistrich *et al.* , 1992). Specifically, chemotherapy drug treatment of childhood cancers can result in varying degrees of gonadotoxicity, which can negatively impact future fertility (Chow *et al.* , 2016, Meirow, 2000). Nevertheless, for many drugs, the magnitude of any potential long-term effect remains to be elucidated, for both males and females, as well as for both adult and pre-pubertal patients. The precise percentage of patients experiencing infertility after cancer therapy, and the degree of this dysfunction, is unknown. In the majority of cases, it is a consequence of spermatogenic impairment for men or premature ovarian failure for women. In the 0-14 years age group, cancer occurs in approximately 1 in 500 children (Cancer Research UK, 2011, www.cancerresearchuk.org, date of access 11/12/2015) and gonadotoxicity for childhood cancer survivors may only become apparent after many years, even decades, of clinical follow-up due to a failure of normal gonadal function in adulthood. The ability to identify agents and regimens that confer a significant risk of gonadal damage will enable patients and their families to make informed decisions regarding the use of available strategies for fertility preservation. Furthermore, understanding the specific mechanisms of action for the effects of different classes of chemotherapeutic drugs on the reproductive system is pivotal to the development of tailored protective tools.

Assessing fertility after chemotherapy is the first step toward any type of investigation into preserving the functionality of the reproductive system. However, in both males and females it is a difficult task that requires long-term follow up and is complicated by the large number of co-existing

4

112   variables in addition to the chemotherapy itself (i.e. type of malignancy, age, and pubertal status).

113   To date, many clinical and experimental studies have increased our knowledge about the degree of

114   ovotoxicity induced by several chemotherapeutic drugs (Gracia *et al.* , 2012, Levine *et al.* , 2015,

115   Waimey *et al.* , 2015). Some studies have been able to identify the specific cellular target of each

116   individual drug in the female gonad and the degree of ovotoxicity that results from exposure (Ben-

117   Aharon and Shalgi, 2012, Meirow, 2000, Meirow *et al.* , 2007, Morgan *et al.* , 2012, Sanders *et al.* ,

118   1996, Thomas-Teinturier *et al.* , 2015) . This information is of particular importance because,

119   although prepubertal females with a good prognosis and high risk of infertility cannot opt for

120   oocyte/embryo cryopreservation as adult women are able to do, they still have the option of ovarian

121   cortical tissue cryopreservation in order to preserve their subsequent fertility (extensively reviewed

122   in Anderson and Wallace, 2011, Levine *et al.*, 2015, Oktay and Oktem, 2009, Waimey *et al.*, 2015,

123   Wallace, 2011). For prepubertal males, it is known that some chemotherapeutic agents impair

124   fertility, however, in many cases, azoospermia is only a temporary outcome and after a variable

125   length of time spermatogenesis recovers (Meistrich, 1986, Schrader *et al.* , 2001). Moreover, much

126   of the knowledge we have about the impact of chemotherapy on spermatogenesis has been

127   obtained from adult patients, with markedly fewer studies about the chemotherapy-induced

128   damage to the reproductive system in male childhood cancer patients, in which spermatogenesis

129   has not yet been established (Bordallo *et al.* , 2004, Meistrich *et al.*, 1992, Nurmio *et al.* , 2009,

130   Wallace *et al.* , 1991). While adult male patients can preserve reproductive potential using the well-

131   established option of sperm cryobanking, for pre-pubertal boys the only potential option is

132   cryopreservation of testicular tissues, a technique that is currently experimental, at the time of

133   writing proven to work only in animal models (Picton *et al.* , 2015).

134

135   Irinotecan is a chemotherapeutic drug commonly administered to both male and female patients

136   and represents the first and second-line therapy for the treatment of metastatic and recurrent

137   colorectal cancer. It is also used in the treatment of several other malignancies, including lymphoma,

5

138   lung, gastrointestinal and pancreatic tumours (Li *et al.* , 2014). In the late 1990s, clinical trials were

139   set up to evaluate its use in paediatric cancers and it is now part of the treatment of refractory solid

140   tumours in young patients (Brennan *et al.* , 2014, Mugishima *et al.* , 2002, Norris *et al.* , 2014).

141   Irinotecan is a water-soluble synthetic version of the alkaloid camptothecin (CPT), initially isolated

142   from the Chinese tree *Camptotheca acuminate*, and then synthesized for medical use as 7-ethyl-10-

143   (4-[1-piperidino]-1piperidino)-carbonyl-camptothecin hydrochloride trihydrate (namely CTP-11 or

144   irinotecan) (Mugishima *et al.*, 2002). Irinotecan works as an S phase-specific inhibitor of

145   topoisomerase I, a key nuclear enzyme for the relaxation of DNA double helix super-coiling during

146   replication (Voigt *et al.* , 1998, Zhang *et al.* , 2004): as such, irinotecan impairs cell proliferation. *In*

147   *vivo*, irinotecan is converted by hydrolysis into its active metabolite, 7-ethyl-10-

148   hydroxycamptothecan (SN38) which is a thousand times more cytotoxic than irinotecan itself

149   (Kawato *et al.* , 1991). Pharmacokinetic studies show plasma concentrations of SN38 in adults

150   ranging between 0.01 and 0.1 $\mu g\ ml^{-1}$ within the first 10 h after CPT-11 administration (Xie, 2002),

151   while plasma concentration of SN38 documented in paediatric patients are around 0.005-0.05 $\mu g\ ml^{-}$

152   ^{1} after infusion of 200mg/m^2 of CPT-11 per day over 3 days (Mugishima *et al.*, 2002). Irinotecan

153   results in a range of severe acute effects, such as diarrhoea, nausea, vomiting and neutropenia;

154   however, the long-term effects of this chemotherapeutic drug metabolite are less clear.

155

156   The effect of irinotecan on fertility has not been described. One research group documented

157   menopausal symptoms in perimenopausal women after administration of irinotecan in combination

158   with other drugs (Tanaka, 2008). However, multiple-agent therapies render it difficult to identify the

159   specific contribution of each individual compound, on top of which menopausal symptomatology

160   can only be an indicative parameter of fertility. Only one study investigated the mechanism of action

161   of irinotecan using a mouse model, showing an increase in granulosa cell (GC) apoptosis after

162   irinotecan administration (Utsunomiya, 2008). However these data are limited to the adult female

163   mouse, with no information regarding the effect on earlier stages of ovarian follicles, particularly

164   those constituting the ovarian reserve. Men undergoing chemotherapy that includes irinotecan are

165   generically advised of a possible subsequent impairment in sperm production (Cancer Research UK,

166   www.cancerresearchuk.org, date of access 28/2/2016), despite the fact that there are no data

167   available regarding the role of irinotecan on spermatogenesis. Most importantly, there is no

168   information available about future fertility following irinotecan administration to prepubertal

169   patients of either gender.

170

171   Using an *in vitro* mouse model, we have previously demonstrated that different classes of

172   chemotherapeutic drugs display variable degrees and mechanisms of ovotoxicity (Lopes *et al.* , 2014,

173   Morgan *et al.* , 2013). Furthermore, since male and female germ cells have undergone markedly

174   different early specialisation supported by different populations of somatic cells, it cannot be

175   assumed that the same drug will have the same effect on male and female gonads. Here, we have

176   used an established mouse ovary culture model and developed an equivalent mouse testis culture

177   model to examine the specific role of irinotecan on prepubertal male and female gonads. For this

178   work, testicular and ovarian tissue were exposed to SN38 *in vitro*, at concentrations spanning those

179   found in the serum of patients treated with irinotecan.

180

181   **Materials and methods**

182

183   *Mice*

184   C57BL/6 mice were kept under 14h:10h light:dark cycle in an approved animal facility, with food and

185   water provided *ad libitum*. Experiments were approved by the University of Edinburgh's Local Ethical

186   Review Committee with procedures performed in accordance with UK Home Office regulations.

187

188   *Testis culture*

189   Testes were collected from post-natal day (pnd) 5 male mice and placed in Leibovitz (L-15,

190   Invitrogen, UK) medium supplemented with 3 µg ml^{-1} of bovine serum albumin (BSA: Sigma-Aldrich

191   Ltd, UK) after the removal of non-gonadal tissue. Each testis was cut into small fragments of roughly

192   0.5 mm^3 using a small scalpel blade (Altomed Ltd, UK), with each piece placed on a floating

193   polycarbonate membrane (Whatman Nucleopore Polycarbonate Membrane, Camlab Ltd,

194   Cambridge, UK) in a 24-well plate (Grenier Bio-one, Stonehouse, UK) containing 1 ml of α-minimum

195   essential media (MEM) culture medium (Invitrogen, UK). As with Sato and colleagues (Sato *et al.*,

196   2011), culture medium was supplemented with 10% knockout serum replacement (KSR, Invitrogen,

197   UK) and incubated under a controlled atmosphere with 5% CO_2 at 37°C for 24 hours (Day 1). On Day

198   2 of culture, vehicle-control 0.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich Ltd, UK) or SN38 (Sigma-

199   Aldrich Ltd, UK) dissolved in DMSO, was added to the medium. Concentrations of SN38 used were:

200   0.002, 0.01, 0.05, 0.1 or 1 µg ml^{-1}, all in 0.5% DMSO. On Day 3, testes were moved into drug-free

201   medium. For cell proliferation experiments, culture medium was supplemented on Day 4 with 15 µg

202   ml^{-1} of bromodeoxyuridine (BrdU; Sigma-Aldrich Ltd, UK) for the final 24h of culture. At the end of

203   Day 4, tissues were fixed and processed as detailed below.

204

205   ***Ovary culture***

206   Ovaries were collected from pnd 4 female mice and placed in L-15 medium supplemented with 3 µg

207   ml^{-1} BSA for removal of non-gonadal tissue. Ovaries were placed on floating polycarbonate

208   membranes in 24-well plates containing α-MEM culture medium supplemented with 3 µg ml^{-1} BSA

209   and incubated under a controlled atmosphere with 5% CO_2 at 37°C for 24 hours (Day 1). On Day 2,

210   medium was supplemented with vehicle-control 0.5% DMSO or increasing concentration of SN38:

211   0.1, 1, 2.5 or 5 µg ml^{-1} in 0.5% DMSO. After 24 hrs (Day 3), ovaries were moved into drug-free

212   medium. For cell proliferation experiments, culture medium was supplemented with 15 µg ml^{-1} BrdU

213   for the final 24h of culture. Ovaries were kept in culture until Day 6, when tissues were processed for

214   analyses as detailed below.

215

216   ***Morphological evaluation***

217   At the end of culture, testes and ovaries were fixed in Bouin's fluid and embedded in paraffin wax.

218   Serial sections of 5 µm thickness were cut and stained with haematoxylin and eosin (H&E).

219   Three testis sections, taken from the beginning, middle and end of each piece of tissue, were

220   photomicrographed (DMLB Leica microscope, Leica Microsystem Ltd, UK) and used for

221   morphological examination by a blind-to-treatment assessor using ImageJ software. In each section,

222   the total number of seminiferous tubules was noted, along with the number of seminiferous tubules

223   that lacked visible germ cells on the basement membrane (Sertoli cell-only tubules). Within each

224   section, the diameter of every spherical tubule was measured. Total section area and seminiferous

225   tubule area were also recorded.

226

227   Every 6th ovarian section was photomicrographed and used for ovarian follicle counts and health

228   assessment using ImageJ software by a blind-to-treatment assessor, as detailed in Morgan et al.

229   (2013). In brief, follicles were staged as: primordial, when an oocyte with a visible germinal vesicle

230   (GV) was surrounded only by flattened GCs; transitional, when an oocyte with a visible GV was

231   surrounded by a mixture of flattened and cuboidal GCs; primary, when an oocyte with a visible GV

232   was surrounded only by cuboidal GCs. Follicles were further classified as unhealthy when containing:

233   an oocyte with eosinophilic and shrunk cytoplasm, and/or condensed nuclear chromatin; GCs, the

234   majority of which were irregularly shaped and/or had condensed chromatin; or those follicles with a

235   combination of unhealthy oocytes and GCs. The total number of follicles was estimated by correcting

236   the count of any visible GV in each section using the Abercrombie formula (Abercrombie, 1946).

237

238   ***Immunofluorescence***

239   At the end of the culture period, ovarian and testis tissue was fixed in 10% neutral buffered formalin

240   (Sigma-Aldrich Ltd, UK) and embedded in paraffin wax. Serial sections of 5 µm thickness were cut

241 and 3 sections, from the beginning, middle and end of each piece of tissue, were used for

242 immunofluorescence as previously described (Lopes *et al.*, 2014). In brief, after rehydration, slides

243 underwent antigen retrieval in sodium citrate (10mM, pH6; Fisher Chemical, Loughborough, UK) and

244 blocking in 20% normal goat serum in PBS (Fisher Scientific UK Ltd, UK) with 0.1% Triton X-100

245 (PBST) and 5% BSA. Primary antibody incubation was performed overnight at 4°C with mouse anti-

246 mouse vasa homologue (MVH; 1:100; Abcam, UK), rabbit anti-cleaved caspase 3 (CC3; 1:500; Cell

247 Signalling Technology, USA) or with rat anti-BrdU (1:200; Abcam, UK) either alone or alongside the

248 antibody for MVH. Slides were incubated for 1h at room temperature with secondary antibodies:

249 Alexa Fluor 568 goat anti-mouse IgG1 (1:200; Invitrogen, UK) for MVH, goat anti-rabbit biotinylated

250 (1:200; DakoCytomation, Denmark) for CC3 and rabbit anti-rat biotinylated (1:200; Vectors Lab, UK)

251 for BrdU, with the latter two reactions followed by 30 min at room temperature with Alexa Fluor 488

252 streptavidin conjugate (1:200; Invitrogen, UK). Slides were counterstained with DAPI (Invitrogen,

253 UK), mounted in Vectashield mounting medium (Vector Laboratories, USA) and photomicrographs

254 obtained (Leica DM5500B microscope on a DFC360FX camera). Image analysis was performed with

255 ImageJ software, with the assessor blind to treatments. The degree of expression of germ cell

256 (MVH), apoptotic (CC3) and proliferation markers (BrdU) was given by the area of immunoreactivity

257 relative to the section area as previously described (Lopes *et al.*, 2014). Randomly selected images

258 were used to compare manual counting of MVH positive cells versus semi-automated analysis using

259 ImageJ software.

260

261 ***Statistical analysis***

262 Statistical analyses of data were performed using GraphPad Prism software (GraphPad Software, Inc,

263 CA, USA). Treatments groups were checked for statistical significance compared to control. The non-

264 parametric Kolmogorov-Smirnov test was used to assess normal distribution. For data with a normal

265 distribution, one-way ANOVA and Bonferroni post-hoc test were applied, while data that were not

266 normally distributed were assessed for statistical significance using the Kruskal-Wallis test followed

10

267   by Dunns post-test. Pearson's correlation coefficient was used to determine the correlation between

268   manual count and automated measurement of the immunofluorescence staining. Results were

269   considered statistically significant where p <0.05.

270

271   **Results**

272

273   ***SN38 impairs testis morphology***

274   In order to assess whether irinotecan affects the morphology of mouse testis, tissue was treated

275   with increasing doses of SN38 (0.002, 0.01, 0.05, 0.1 or 1 μg ml^{-1}) and histological sections were

276   assessed (Fig.1A). Seminiferous cord structure was maintained across the experimental groups, but

277   examination of the histological sections shows that highest concentration of SN38 led to a dramatic

278   increase in Sertoli cell-only tubules. While the density of seminiferous tubules was unaffected by

279   treatment (Fig.1Bi), seminiferous tubule diameter decreased significantly at the highest SN38

280   concentration (Fig.1Bii), along with a dose-response increase in the number of Sertoli cell-only

281   tubules that reached significance at 0.1 and 1 μg ml^{-1} SN38, rising from around 2% in control to 15%

282   and 52% of tubules respectively (Fig.1Biii).

283

284   ***Manual counting validates semi-automated measurement of immunoreactivity***

285   To compare manual counting versus semi-automated measurement of immunofluorescence cells, 3-

286   4 randomly selected testis sections immunostained for MVH for each experimental group (control,

287   0.002, 0.01, 0.05, 0.1 or 1 μg ml^{-1} SN38) were analysed using both systems (Fig.2). Manual counts of

288   MVH positive cells per section showed a significant decrease in germ cell number with a reduction

289   from 134 cells per 100 mm^2 in control group to 75, 55 and 6 cells per 100 mm^2 in 0.05, 0.1 or 1 μg ml^{-}

290   ^{1} SN38 groups, respectively (Fig.2A). A similar decrease was demonstrated with the semi-automated

291   measurement, where the percentage of section area expressing MVH immunostaining was reduced

292   by 15% in control group to 8%, 4% and 0.9% in the 0.05, 0.1 or 1 μg ml^{-1} SN38 groups respectively

11

293   (Fig.2B). Both measurement systems showed that germ cell number and percentage immunostained

294   area decreased by approximately 2, 3 and 20 fold in the groups treated with the highest SN38

295   concentration compared with control, demonstrating a highly significant correlation between the

296   two methods, (r=0.954, p<0.0001; Fig.2C).

297

298   ***SN38 affects testicular germ cell number and proliferation, but not apoptosis***

299   Immunohistochemistry was used to assess whether SN38 affected the number of germ cells (MVH

300   expression; Fig.3A), the amount of apoptosis (CC3 expression; Fig.3B) or the level of cell proliferation

301   (BrdU expression; Fig.3C). MVH-expression, correlating with germ cell numbers, decreased markedly

302   in a dose dependent manner, reaching statistical significance at concentrations of 0.1 and 1 µg ml^{-1}

303   SN38 when compared with the control group (Fig.3Aiii), with MVH-expression falling from 13.9% to

304   4.3% and 0.4% of tubule area, respectively. No difference was observed in the percentage of CC3-

305   positive, apoptotic cells across treatments (Fig.3Biii). There was a decrease in the percentage of

306   proliferating cells within tubules, becoming significantly lower after exposure to 0.1 and 1 µg ml^{-1}

307   SN38 (Fig.3Ciii), with the BrdU expression falling from 20.5% to 13.4% and 12.8% of the tubule,

308   respectively.

309

310   Double immunofluorescence for MVH and BrdU was carried out on randomly selected sections from

311   control tissue and from tissue exposed to the two highest concentrations of SN38, 0.1 and 1 µg ml^{-1},

312   to determine if any of the spermatogonia remaining were still proliferating (Fig.4). In the control

313   group, the majority of the germ cells were actively proliferating, but, even at the highest

314   concentration of SN38, with very few germ cells remaining, occasional proliferative germ cells could

315   still be found (Fig.4).

316

317   ***Ovarian follicle morphology is unaffected by SN38***

318   To assess whether the ovarian follicle reserve was affected by exposure to SN38, neonatal mouse

319   ovaries were cultured in the presence or absence of SN38. Initial experiments exposing ovaries to

320   concentrations up to the highest levels found in the plasma of adult patients treated with irinotecan

321   (0.1μg ml^{-1}) found no evidence of damage (data not shown). In order to determine if a dose-

322   response pattern could be found, concentrations were therefore increased markedly, exposing

323   ovaries to 0.1, 1, 2.5 or 5 μg ml^{-1} of SN38. Overall, effects of SN38 on the ovary were not observed

324   until the concentration of SN38 was much higher than the highest concentrations found in the

325   plasma of patients treated with irinotecan, and even then effects observed were not marked (Fig.5).

326   Examination of histological sections (Fig.5Ai-iii) revealed signs of stromal cell damage only after

327   exposure to 5 μg ml^{-1} SN38. Total follicle number was unaffected by drug treatment (Fig.5Bi), while

328   the percentage of follicles assessed as unhealthy was significantly increased only in the 2.5 μg ml^{-1}

329   SN38 treatment group, rising from 23% to 40% (Fig.5Bii). To determine whether a specific stage of

330   early follicle development was affected by SN38 treatment, the percentage of follicles assessed as

331   unhealthy was examined separately for primordial, transitional and primary follicles (Fig.5C): only

332   primary follicles exposed to 1 μm ml^{-1} SN38 showed a significant increase in the percentage deemed

333   to be unhealthy, rising from 27% to 57% (Fig.5Ciii).

334

335   ***SN38 does not affect ovarian germ cell number, ovarian cell apoptosis or proliferation***

336   As with the testis, the effect of SN38 exposure on germ cell number, apoptosis and proliferation was

337   assessed using immunohistochemistry, by examining expression of the germ cell marker MVH

338   (Fig.6A), apoptotic cell marker CC3 (Fig.6B) and proliferation marker BrdU (Fig.6C). Relative to

339   section area, the area of immunoreactivity of all three markers (MVH, CC3 and BrdU) was unaffected

340   by the drug, even after exposure to 5 μm ml^{-1} SN38 (Fig.6).

341

342   **Discussion**

343

344   The present study shows that SN38, the active metabolite of irinotecan, is cytotoxic to male germ

345   cells in the prepubertal mouse testis, whilst no similar effect was demonstrated in the prepubertal

346   ovary. An *in vitro* mouse model was used to expose male and female gonadal tissue to

347   concentrations of SN38 that are clinically relevant, covering the range of reported concentrations

348   found in the serum of patients shortly after administration of irinotecan. Results show that the

349   prepubertal testis is affected by exposure to the high end of clinically relevant concentrations of

350   SN38 (0.1 µg ml^{-1} SN38 and above). In contrast, there was little effect of SN38 on the ovary even

351   when concentrations were increased to 50-fold higher concentrations of SN38 that have been found

352   in patients.

353

354   To the best of our knowledge, there is no published information about the effect of SN38 on the

355   prepubertal testis or ovary. Work here shows that SN38 targeted spermatogonia, significantly and

356   markedly reducing germ cell number, leading to a 3.5-fold and 35-fold reduction in MVH protein

357   expression after exposure to 0.1 and 1 µg ml^{-1} SN38 respectively, accompanied by an equally

358   dramatic 7.5- and 26-fold increase in Sertoli cell-only tubules. SN38 is a potent inhibitor of cell

359   proliferation (Bomgaars *et al.* , 2006). It is believed that the high growth rate of the germ cell

360   population renders the testis particularly sensitive to chemotherapeutic drugs whose principal

361   mechanism of action is to impair the replication ability of cancer cells. Here, germ cells decreased

362   significantly in tubules of testis tissue exposed to high SN38 concentrations, as did all proliferating

363   cells in the seminiferous tubule. However, the high proliferation rate may not be the only

364   explanation for the specific vulnerability of spermatogonial germ cells, since other factors could also

365   be involved. This could include factors intrinsic to the germ cells or indirect actions via somatic cells

366   that impair signalling to germ cells, resulting in germ cell loss. Indirect effects on spermatogonial

367   proliferation and differentiation via the somatic Sertoli cells have been shown (Brilhante *et al.* ,

368   2012). For example, KIT-ligand (also known as stem cell factor) produced by Sertoli cells is required

369   to support differentiation of spermatogonia through interaction with the c-kit receptor on

14

370   differentiated spermatogonia (Ohta *et al.* , 2000). In addition, irradiation in the rat testis has been

371   demonstrated to block spermatogonial differentiation as a result of damage to the somatic

372   compartment (Zhang *et al.* , 2007). Results here suggest that chemotherapeutic drugs have

373   differential effects on germ cells rather than Sertoli cells in terms of cell loss within the seminiferous

374   tubules; however, effects of SN38 exposure on other testis somatic cell types (e.g. Leydig or

375   peritubular myoid cells) require further investigation.

376

377   Whilst the present study has demonstrated clear and dramatic effects of SN38 on the germ cells of

378   the prepubertal mouse testis, extrapolation of these results to prepubertal human must take into

379   account variations in spermatogonial development between these species. The germ cell population

380   in rodents arises from spermatogonial stem cells (SSCs) that are generally classified as A_{single}, which

381   regularly, although infrequently, self-renew and A_{paired}/$A_{aligned}$ which are committed progenitor cells

382   with only a few cycles of self-renewing divisions (Hermann *et al.* , 2010). In primates, including

383   humans, two separate spermatogonial cell populations make up the SSC pool: a stem cell reserve

384   with a low/none proliferative activity under physiological conditions (A_{dark}) and a separate functional

385   pool of highly proliferative progenitors (A_{pale}) (Mitchell *et al.* , 2009). However, functional and

386   molecular similarities and/or differences between human and rodent SSCs are still to be fully

387   elucidated, mainly because of the lack of information about the regulation of human SSCs (Nagano

388   *et al.* , 2002, Wu *et al.* , 2009). As such, species-specific differences in sensitivity to cytotoxic agents

389   would require further investigation, possibly by using an experimental animal species more closely

390   related to humans. As with all *in vitro* work, it will be important to perform *in vivo* studies to further

391   validate our findings. These results provide valuable information about specific effects of exposures,

392   based on dosage and timing, able to inform any future in vivo studies.

393

394   In addition, the present model investigates the effect of SN38 at 4-6 days after a single exposure, as

395   opposed to administration over several cycles, as in the regimens used in patients. In this respect,

15

396 the present results may underestimate the fertility impairment that may occur after repeated

397 Irinotecan administration to young male cancer patients (Ise *et al.* , 1986, Nurmio *et al.*, 2009).

398 Importantly, amongst the very few remaining spermatogonial cells exposed to high SN38

399 concentrations, some retained their proliferative ability, leaving open the possibility that the

400 seminiferous tubules could be repopulated over time, giving the potential for future fertility.

401

402 It has been proposed that male gonads are more sensitive to chemotherapy than female gonads

403 (Ajala *et al.* , 2010). Work here has also evaluated the impact of SN38 on the ovarian follicle reserve

404 and on early stages of follicle development. Damage to these quiescent/early growing ovarian

405 follicles can have a major impact on the subsequent reproductive capability of females, particularly

406 on long-term fertility. However, data here suggest that irinotecan administration is unlikely to

407 impact on female fertility. No sign of damage was observed when mouse ovarian tissue was exposed

408 to concentrations of SN38 comparable to those found in cancer patients. Even when SN38

409 concentrations were further increased to up to 50-fold higher concentrations than those found in

410 patient plasma, there was little effect, with no morphological changes evident in primordial or early

411 growing follicles, and with apoptosis and cell proliferation unaffected by drug exposure. Utsunomiya

412 et al. (2008) also failed to find damage in small or medium follicles in response to irinotecan

413 administration in mice, although apoptosis of GCs was observed in larger follicles. Here, it is

414 impossible to exclude the possibility that SN38 has produced subtle damage to early-stage follicles,

415 the effects of which might not become apparent until later developmental stages, but the simplest

416 and most parsimonious hypothesis is that early ovarian follicle stages are not sensitive to SN38

417 insult.

418

419 Work here used a new tissue culture system to support short-term survival and development of the

420 early neonatal testis *in vitro*, modified from ovary culture systems used by our laboratories (Lopes *et*

421 *al.*, 2014, Morgan *et al.*, 2013) and other testis culture systems (Sato *et al.*, 2011). Examination of

16

422 the control photomicrographs and data show that the culture system maintains the structural

423 integrity of the seminiferous tubules, and that the tubules contain proliferating germ and somatic

424 cells.

425

426 In summary, we have investigated the gonadotoxicity of irinotecan, a chemotherapeutic drug widely

427 administered to patients of both sexes and of all ages, pre-pubertal and adult. Results show that the

428 irinotecan-metabolite SN38 results in damage to testes, significantly affecting germ cells following

429 exposure to clinically relevant concentrations of SN38, in contrast to only minor effects on the ovary,

430 with no effects on germ cell number even following exposure to 50 times higher concentrations of

431 SN38 than those reported to date in patients following irinotecan administration. As such, our

432 results using a prepubertal mouse model could indicate that germ cells in the ovaries of prepubertal

433 girls may be less susceptible to damage by irinotecan administration than those in the testes of

434 prepubertal boys; however, further studies, using non-human primate or human models, are

435 necessary to confirm these results. In addition, an examination of long-term effects of

436 irinotecan/SN38, and investigation into ways of protecting against damage, is of major importance.

437 The retention of some proliferative germ cells after exposure to SN38 does allow for the possibility

438 of germ cells repopulating the seminiferous tubules, which could in turn lead to recovery of fertility,

439 although this will require further study. More generally, our results highlight the fact that

440 chemotherapy drugs can have differential effects on the gonads of males and females.

441

442 **Acknowledgements**

444

445 **Authors' roles**

446 FL, RTM and NS designed experiments; FL and RS performed experiments; FL, RTM, and NS

447 interpreted data; FL and NS wrote the manuscript; RS and RTM critically revised the manuscript.

17

448

452

Conflict of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of the

present research.

456

457

458

459

460

461 **References**

462

463   Abercrombie M. Estimation of nuclear population from microtome sections. The Anatomical record.
464   1946;**94**:239-47.
465   Ajala T, Rafi J, Larsen-Disney P, Howell R. Fertility preservation for cancer patients: a review.
466   Obstetrics and gynecology international. 2010;**2010**:160386.
467   Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WH. Cancer treatment and
468   gonadal function: experimental and established strategies for fertility preservation in children and
469   young adults. The lancet Diabetes & endocrinology. 2015;**3**:556-67.
470   Anderson RA, Wallace WH. Fertility preservation in girls and young women. Clinical endocrinology.
471   2011;**75**:409-19.
472   Ben-Aharon I, Shalgi R. What lies behind chemotherapy-induced ovarian toxicity? Reproduction.
473   2012;**144**:153-63.
474   Bomgaars L, Kerr J, Berg S, Kuttesch J, Klenke R, Blaney SM. A phase I study of irinotecan
475   administered on a weekly schedule in pediatric patients. Pediatric blood & cancer. 2006;**46**:50-5.
476   Bordallo MA, Guimaraes MM, Pessoa CH, Carrico MK, Dimetz T, Gazolla HM, Dobbin J, Castilho IA.
477   Decreased serum inhibin B/FSH ratio as a marker of Sertoli cell function in male survivors after
478   chemotherapy in childhood and adolescence. Journal of pediatric endocrinology & metabolism :
479   JPEM. 2004;**17**:879-87.
480   Brennan RC, Furman W, Mao S, Wu J, Turner DC, Stewart CF, Santana V, McGregor LM. Phase I dose
481   escalation and pharmacokinetic study of oral gefitinib and irinotecan in children with refractory solid
482   tumors. Cancer chemotherapy and pharmacology. 2014;**74**:1191-8.
483   Brilhante O, Okada FK, Sasso-Cerri E, Stumpp T, Miraglia SM. Late morfofunctional alterations of the
484   Sertoli cell caused by doxorubicin administered to prepubertal rats. Reproductive biology and
485   endocrinology : RB&E. 2012;**10**:79.
486   Chow EJ, Stratton KL, Leisenring WM, Oeffinger KC, Sklar CA, Donaldson SS, Ginsberg JP, Kenney LB,
487   Levine JM, Robison LL *et al.* Pregnancy after chemotherapy in male and female survivors of
488   childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor
489   Study cohort. The Lancet Oncology. 2016.
490   Gracia CR, Sammel MD, Freeman E, Prewitt M, Carlson C, Ray A, Vance A, Ginsberg JP. Impact of
491   cancer therapies on ovarian reserve. Fertility and sterility. 2012;**97**:134-40.e1.
492   Hermann BP, Sukhwani M, Hansel MC, Orwig KE. Spermatogonial stem cells in higher primates: are
493   there differences from those in rodents? Reproduction. 2010;**139**:479-93.
494   Ise T, Kishi K, Imashuku S, Tsukada M, Tsukimoto I, Tsujino G, Bessho F, Tanaka H, Miyazaki S, Sakurai
495   M *et al.* Testicular histology and function following long-term chemotherapy of acute leukemia in
496   children and outcome of the patients who received testicular biopsy. The American journal of
497   pediatric hematology/oncology. 1986;**8**:288-93.
498   Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracellular roles of SN-38, a metabolite of the
499   camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer research. 1991;**51**:4187-
500   91.
501   Levine JM, Kelvin JF, Quinn GP, Gracia CR. Infertility in reproductive-age female cancer survivors.
502   Cancer. 2015;**121**:1532-9.
503   Li XX, Zheng HT, Peng JJ, Huang LY, Shi DB, Liang L, Cai SJ. RNA-seq reveals determinants for
504   irinotecan sensitivity/resistance in colorectal cancer cell lines. International journal of clinical and
505   experimental pathology. 2014;**7**:2729-36.
506   Lopes F, Smith R, Anderson RA, Spears N. Docetaxel induces moderate ovarian toxicity in mice,
507   primarily affecting granulosa cells of early growing follicles. Molecular human reproduction.
508   2014;**20**:948-59.
509   Meirow D. Reproduction post-chemotherapy in young cancer patients. Molecular and cellular
510   endocrinology. 2000;**169**:123-31.

511 Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, Raanani H, Levron J, Fridman E. Cortical
512 fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential
513 mechanisms of ovarian injury. Human reproduction (Oxford, England). 2007;**22**:1626-33.
514 Meistrich ML. Relationship between spermatogonial stem cell survival and testis function after
515 cytotoxic therapy. The British journal of cancer Supplement. 1986;**7**:89-101.
516 Meistrich ML. Effects of chemotherapy and radiotherapy on spermatogenesis in humans. Fertility
517 and sterility. 2013;**100**:1180-6.
518 Meistrich ML, Wilson G, Brown BW, da Cunha MF, Lipshultz LI. Impact of cyclophosphamide on long-
519 term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft
520 tissue sarcomas. Cancer. 1992;**70**:2703-12.
521 Mitchell RT, Saunders PT, Sharpe RM, Kelnar CJ, Wallace WH. Male fertility and strategies for fertility
522 preservation following childhood cancer treatment. Endocrine development. 2009;**15**:101-34.
523 Morgan S, Anderson RA, Gourley C, Wallace WH, Spears N. How do chemotherapeutic agents
524 damage the ovary? Human reproduction update. 2012;**18**:525-35.
525 Morgan S, Lopes F, Gourley C, Anderson RA, Spears N. Cisplatin and doxorubicin induce distinct
526 mechanisms of ovarian follicle loss; imatinib provides selective protection only against cisplatin. PloS
527 one. 2013;**8**:e70117.
528 Mugishima H, Matsunaga T, Yagi K, Asami K, Mimaya J, Suita S, Kishimoto T, Sawada T, Tsuchida Y,
529 Kaneko M. Phase I study of irinotecan in pediatric patients with malignant solid tumors. Journal of
530 pediatric hematology/oncology. 2002;**24**:94-100.
531 Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse
532 testes. Fertility and sterility. 2002;**78**:1225-33.
533 Norris RE, Shusterman S, Gore L, Muscal JA, Macy ME, Fox E, Berkowitz N, Buchbinder A, Bagatell R.
534 Phase 1 evaluation of EZN-2208, a polyethylene glycol conjugate of SN38, in children adolescents
535 and young adults with relapsed or refractory solid tumors. Pediatric blood & cancer. 2014;**61**:1792-7.
536 Nurmio M, Keros V, Lahteenmaki P, Salmi T, Kallajoki M, Jahnukainen K. Effect of childhood acute
537 lymphoblastic leukemia therapy on spermatogonia populations and future fertility. The Journal of
538 clinical endocrinology and metabolism. 2009;**94**:2119-22.
539 Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in
540 spermatogonial stem cells: the role of c-kit and its ligand SCF. Development (Cambridge, England).
541 2000;**127**:2125-31.
542 Oktay K, Oktem O. Fertility preservation medicine: a new field in the care of young cancer survivors.
543 Pediatric blood & cancer. 2009;**53**:267-73.
544 Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G,
545 Rives N, Tournaye H *et al.* A European perspective on testicular tissue cryopreservation for fertility
546 preservation in prepubertal and adolescent boysdagger. Human reproduction (Oxford, England).
547 2015;**30**:2463-75.
548 Sanders JE, Hawley J, Levy W, Gooley T, Buckner CD, Deeg HJ, Doney K, Storb R, Sullivan K,
549 Witherspoon R *et al.* Pregnancies following high-dose cyclophosphamide with or without high-dose
550 busulfan or total-body irradiation and bone marrow transplantation. Blood. 1996;**87**:3045-52.
551 Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T. In vitro production of
552 functional sperm in cultured neonatal mouse testes. Nature. 2011;**471**:504-7.
553 Schrader M, Muller M, Straub B, Miller K. The impact of chemotherapy on male fertility: a survey of
554 the biologic basis and clinical aspects. Reproductive toxicology (Elmsford, NY). 2001;**15**:611-7.
555 Tanaka TU, T; Utsunomya H, H; Umesaki, N. Irinotecan HCL, an anticancer topoisomerase I inhibitor,
556 frequently induces ovarian failure in premenopausal and perimenopausal women Oncology reports.
557 2008;**19**:1123-33.
558 Thomas-Teinturier C, Allodji RS, Svetlova E, Frey MA, Oberlin O, Millischer AE, Epelboin S, Decanter
559 C, Pacquement H, Tabone MD *et al.* Ovarian reserve after treatment with alkylating agents during
560 childhood. Human reproduction (Oxford, England). 2015;**30**:1437-46.

561 Utsunomiya TT, T; Utsunomya, H; Umesaki N. A novel molecular mechanism for anticancer drug-
562 indiced ovarian failure: Irinotecan HCl, an anticancer topoisomerase I inhibitor, induces specific FasL
563 expression in granulosa cells of large ovarian follicles to enhance follicular apoptosis. International
564 Journal of Oncology. 2008;**32**:991-1000.
565 Voigt W, Matsui S, Yin MB, Burhans WC, Minderman H, Rustum YM. Topoisomerase-I inhibitor SN-38
566 can induce DNA damage and chromosomal aberrations independent from DNA synthesis. Anticancer
567 research. 1998;**18**:3499-505.
568 Waimey KE, Smith BM, Confino R, Jeruss JS, Pavone ME. Understanding Fertility in Young Female
569 Cancer Patients. Journal of women's health. 2015;**24**:812-8.
570 Wallace WH. Oncofertility and preservation of reproductive capacity in children and young adults.
571 Cancer. 2011;**117**:2301-10.
572 Wallace WH, Shalet SM, Lendon M, Morris-Jones PH. Male fertility in long-term survivors of
573 childhood acute lymphoblastic leukaemia. International journal of andrology. 1991;**14**:312-9.
574 Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, Ginsberg JP, Brinster RL.
575 Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of
576 germline stem cell regulatory molecules. Proceedings of the National Academy of Sciences of the
577 United States of America. 2009;**106**:21672-7.
578 Xie R. Clinical Pharmacokinetics of Irinotecan and Its Metabolites: A Population Analysis. Journal of
579 Clinical Oncology. 2002;**20**:3293-301.
580 Zhang JA, Xuan T, Parmar M, Ma L, Ugwu S, Ali S, Ahmad I. Development and characterization of a
581 novel liposome-based formulation of SN-38. International journal of pharmaceutics. 2004;**270**:93-
582 107.
583 Zhang Z, Shao S, Meistrich ML. The radiation-induced block in spermatogonial differentiation is due
584 to damage to the somatic environment, not the germ cells. Journal of cellular physiology.
585 2007;**211**:149-58.

586

587

Figure legends

Figure 1. Exposure of mouse testis to SN38 affects tissue morphology. Testis tissue exposed *in vitro* to varying concentrations of the irinotecan metabolite 7-ethyl-10-hydroxycamptothecan (SN38: 0.002, 0.01, 0.05, 0.1, 1 µg ml^{-1}, all in 0.5% dimethylsulphoxide (DMSO) as diluent) were processed for morphological examination. **A:** Representative photomicrographs of haematoxylin and eosin (H&E) stained sections from 3 experimental groups: control (**Ai**), middle (**Aii**) and highest (**Aiii**) SN38 concentrations. Insets are higher magnification of framed areas. The highest SN38 concentration caused a dramatic increase in Sertoli cell-only tubules (arrowhead). **B:** SN38 treatment did not affect the density of seminiferous tubules (**Bi**). Tubule diameter was significantly smaller in testis exposed to the highest SN38 concentration (**Bii**); and the number of Sertoli cell-only tubules increased in the two highest SN38 concentrations (**Biii**). A minimum of 3 pieces were analysed for each treatment and for the corresponding control (in 0.5% DMSO). Scale bars = 50 µm. Graphs show mean ± SEM. Data were analysed using Kruskal-Wallis test followed by Dunns post-test; ** $p < 0.001$, *** $p < 0.001$ versus control.

Figure 2. Validation of the semi-automated measurement of immunofluorescence. Immunostained sections for the germ cell marker mouse vasa homologue (MVH) were randomly selected from each experimental group and analysed using both the manual counting of germ cells and the percentage of the section area expressing the germ cell marker. A strong correlation is present between the number of germ cells per 100 mm^2 (**A**) and the percentage of stained area (**B**) across all the experimental groups (r = 0.954, $p < 0.0001$) (**C**). Histograms show mean ± SEM. Data were analysed using Kruskal-Wallis test followed by Dunns post-test (**A**, **B**) and by Pearson's correlation coefficient (**C**); * $p < 0.05$, **** $p < 0.0001$ versus control.

614 **Figure 3. Seminiferous tubules of mouse testes treated with SN38 contain fewer germ cells and**

615 **proliferating cells.** Testis tissue exposed *in vitro* to varying concentrations of SN38 (0.002, 0.01,

616 0.05, 0.1, 1 µg ml^{-1}) were analysed for the protein expression of: (**A**) germ cell marker MVH (red), (**B**)

617 apoptotic marker cleaved Caspase 3 (CC3; green), and (**C**) proliferation marker bromodeoxyuridine

618 (BrdU; green). Sections were counterstained with DAPI (blue). Photomicrographs of representative

619 immunostained sections from control (**Ai, Bi, Ci**) and 0.1 µg ml^{-1} SN38 (**Aii, Bii, Cii**) groups. Insets are

620 higher magnification of framed areas. Graphs show protein expression calculated as a percentage of

621 tubule (**Aiii** and **Ciii**) or section (**Biii**) area. MVH and BrdU expression dramatically declined at the two

622 highest SN38 concentrations (**Aiii** and **Ciii**), while CC3 is unaffected by drug treatment (**Biii**). The

623 number of testis pieces analysed for each experimental group were between 5 and 9 in the MVH and

624 CC3 assessments and 5-8 in the BrdU staining, all from at least 3 independent experiments. Scale

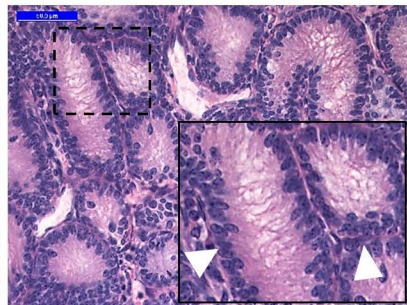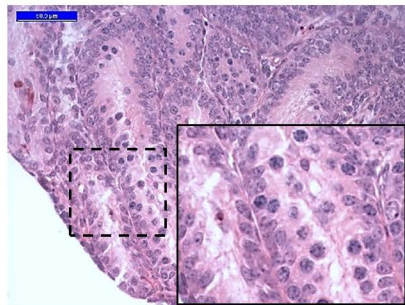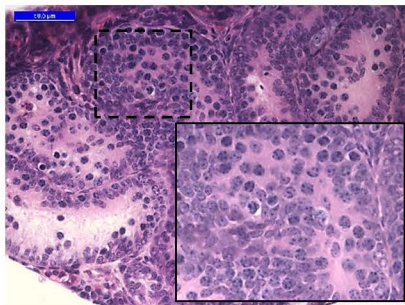625 bars = 100 µm. Graphs show mean ± SEM. Data were analysed using Kruskal-Wallis test followed by

626 Dunns post-test; * $p < 0.05$, ** $p < 0.01$ versus control.

627

628 **Figure 4. Proliferative germ cells are still present in mouse testis treated with SN38.** Testis tissue

629 exposed *in vitro* to 0.1 or 1 µg ml^{-1} SN38 were analysed for co-expression of germ cell marker MVH

630 (red) and proliferation marker BrdU (green), with DAPI (blue) used as counterstain.

631 Photomicrographs of representative immunostained sections from control (**A**), 0.1 µg ml^{-1} (**B**) and 1

632 µg ml^{-1} SN38 (**C**) groups. Insets are magnification of framed areas. The number of proliferating germ

633 cells decreased in treated tissues, but they were still occasionally found even at in the highest SN38

634 concentration group (arrowheads). Scale bars = 50 µm.

635

636 **Figure 5. Exposure of mouse ovaries to SN38 does not impair tissue morphology.** Whole ovaries

637 exposed *in vitro* to high concentrations of SN38 (0.1, 1, 2.5, 5 µg ml^{-1}) were processed for

638 morphological examination and follicle count. Representative photomicrographs of H&E stained

639 sections from 3 experimental groups: control (**Ai**), lowest (**Aii**) and highest (**Aiii**) SN38

23

640   concentrations. Insets are magnification of framed areas. Follicles with an unhealthy oocyte (black

641   arrowhead) or unhealthy granulosa cells (white arrowhead), as well as degenerated stroma cells

642   (arrow) were more often present in the highest SN38 group (**Aiii**). SN38 did not affect total ovarian

643   follicle number (**Bi**) and only 2.5 µg ml^{-1} SN38 concentration increased the percentage of unhealthy

644   follicles (**Bii**). SN38 did not affect the percentage of unhealthy primordial or transitional follicles (**Ci**

645   and **Cii**) whilst the number of unhealthy primary follicles was significantly increased only following

646   treatment with 1 µg ml^{-1} SN38 (**Ciii**). The number of ovaries analysed was between 8 and 12 for each

647   treatment/control group. Graphs show mean ± SEM. Data were analysed using one-way ANOVA

648   followed by Bonferroni post-hoc test; * $p < 0.05$ versus control. Scale bars = 50 µm.

649

650   **Figure 6 SN38 does not affect the expression of germ cell, apoptosis and proliferation markers in**

651   **the mouse ovary.** Whole ovaries exposed *in vitro* to high concentrations of SN38 (0.1, 1, 2.5, 5 µg

652   ml^{-1}) were analysed for the expression of: (**A**) the germ cell marker MVH, (**B**) the apoptotic marker

653   CC3; and the (**C**) proliferative marker BrdU. Photomicrographs are representative of sections from

654   control (**Ai, Bi, Ci**) and 0.1 µg ml^{-1} SN38 concentration (**Aii, Bii, Cii**) groups. Graphs show MVH (**Aiii**),

655   CC3 (**Biii**) and BrdU (**Diii**) expression levels as a percentage of section area: expression was

656   unaffected by SN38 treatment in all cases. A minimum of 4 ovaries were analysed in each

657   experimental group in MVH and CC3 assessments and 5 ovaries in the BrdU staining, all from at least

658   3 independent experiments. Scale bars = 100 µm. Graphs show mean ± SEM. Data were analysed

659   using one-way ANOVA.

660

24

Testis

Control   **0.05µgml⁻¹ SN38**   **1µgml⁻¹ SN38**

Ai   **Aii**   **Aiii**

Bi — % of section area covered by seminiferous tubules; x-axis: Control, 0.002, 0.01, 0.05, 0.1, 1; SN38 (µgml⁻¹)

Bii — Seminiferous tubule diameter; x-axis: Control, 0.002, 0.01, 0.05, 0.1, 1; SN38 (µgml⁻¹); ** on 1

Biii — % of Sertoli cells-only tubules relative to tubules; x-axis: Control, 0.002, 0.01, 0.05, 0.1, 1; SN38 (µgml⁻¹); ** on 0.1, *** on 1
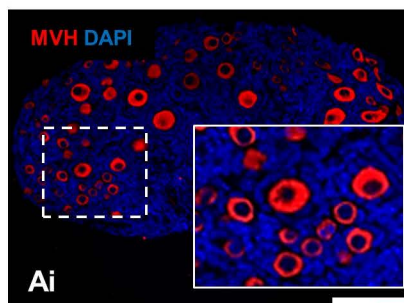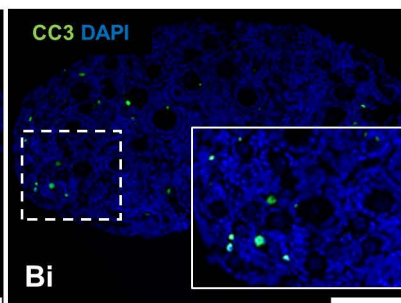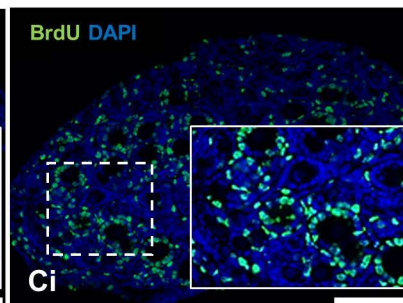
A

B

C
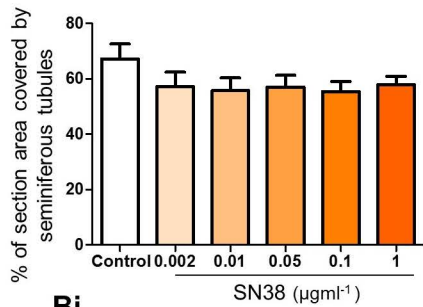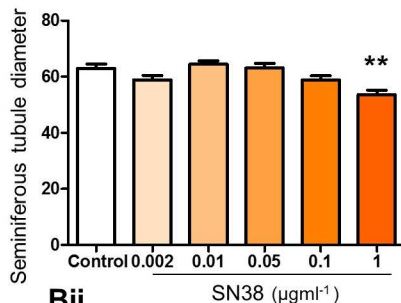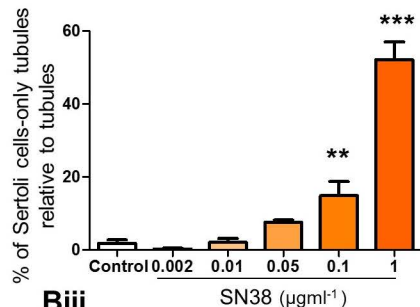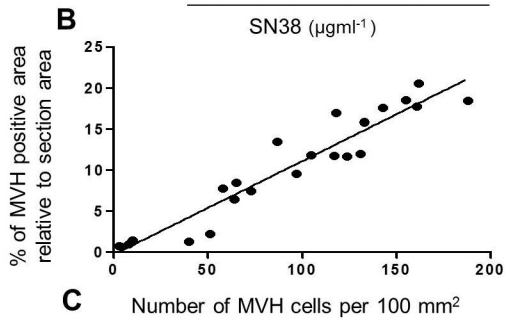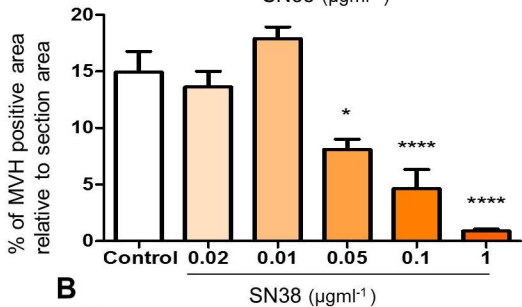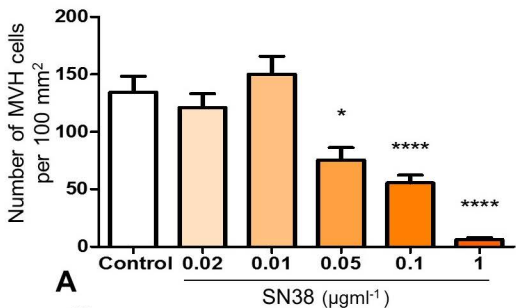
Testis

A: MVH

B: CC3

C: BrdU
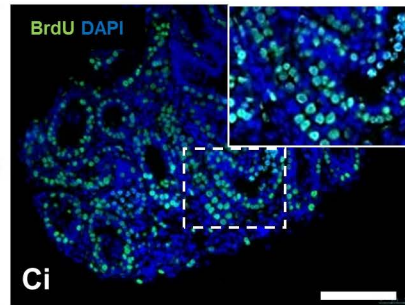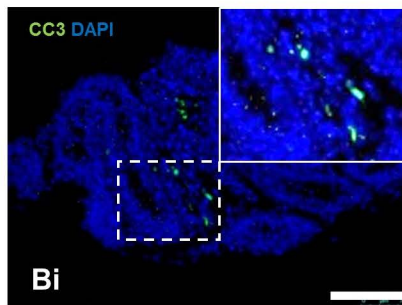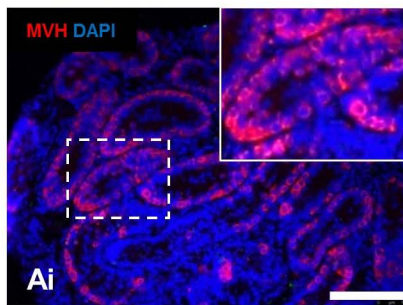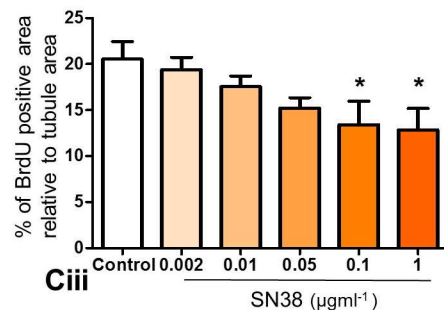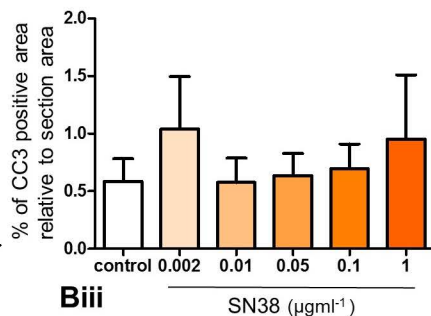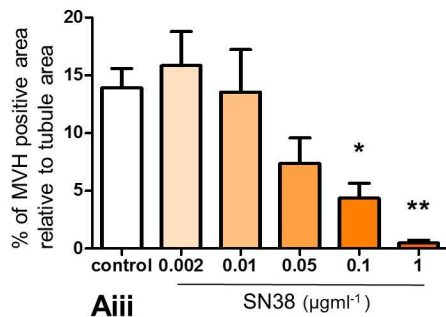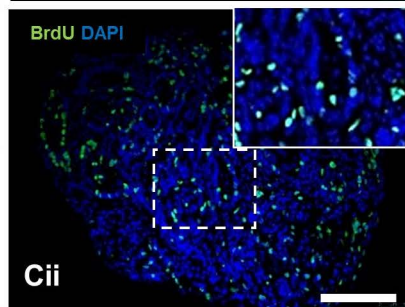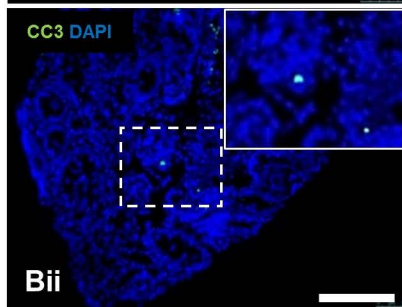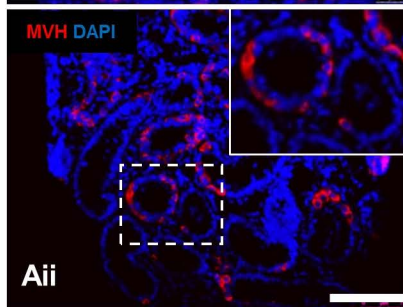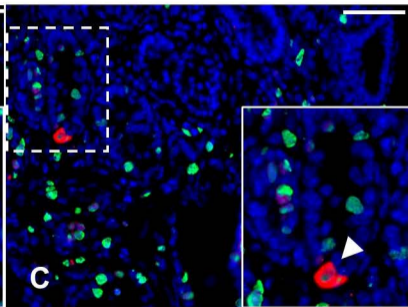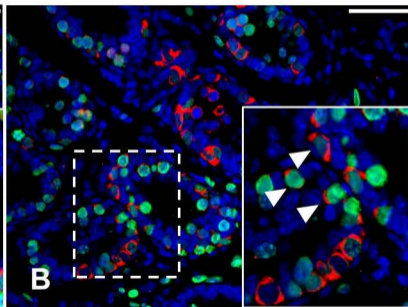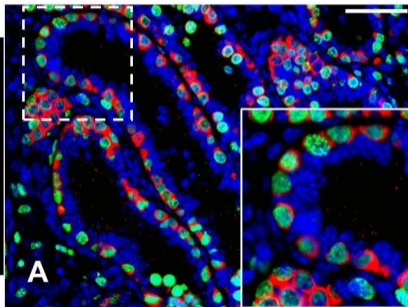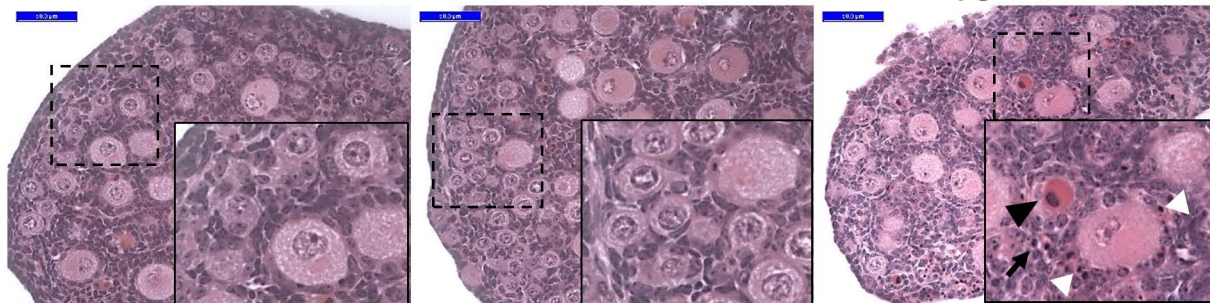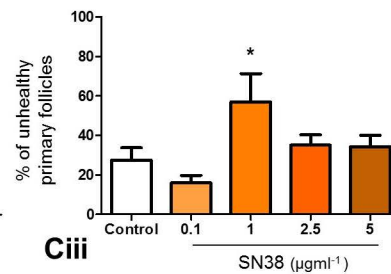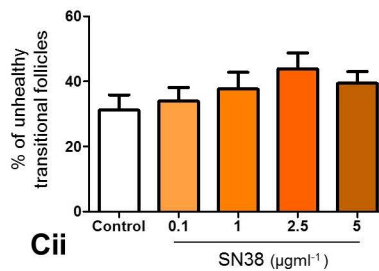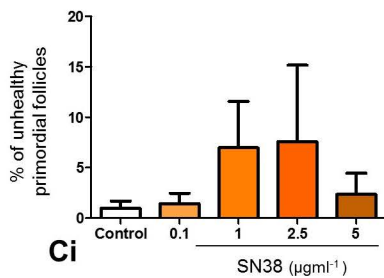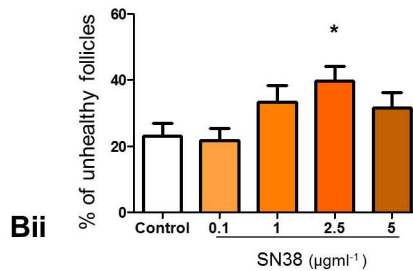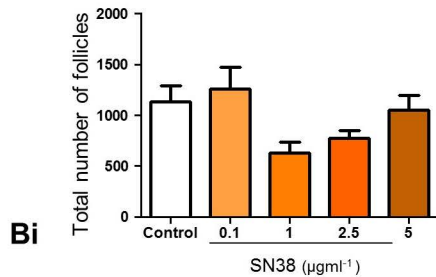
Control

Ai
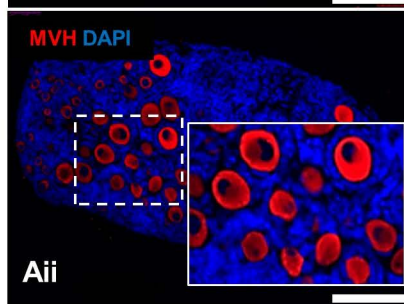
Bi

Ci

0.1µgml-1 SN38

Aii

Bii

Cii

Aiii — % of MVH positive area relative to tubule area vs SN38 (µgml⁻¹): control, 0.002, 0.01, 0.05, 0.1 *, 1 **

Biii — % of CC3 positive area relative to section area vs SN38 (µgml⁻¹): control, 0.002, 0.01, 0.05, 0.1, 1

Ciii — % of BrdU positive area relative to tubule area vs SN38 (µgml⁻¹): Control, 0.002, 0.01, 0.05, 0.1 *, 1 *

Control   **0.1μgml⁻¹ SN38**   **1μgml⁻¹ SN38**

MVH BrdU DAPI
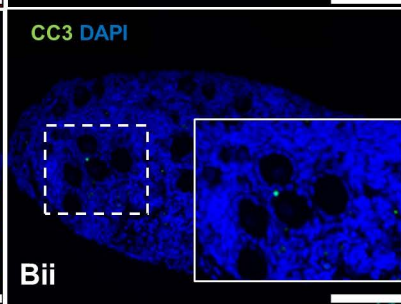
A   B   C

Ovary

Ovary

A: MVH   B: CC3   C: BrdU

Control

Ai — MVH DAPI

Bi — CC3 DAPI

Ci — BrdU DAPI

0.1 µgml⁻¹ SN38

Aii — MVH DAPI

Bii — CC3 DAPI

Cii — BrdU DAPI

Aiii — % of MVH positive area relative to section area — Control, SN38 (μgml^{-1}): 0.1, 1, 2.5, 5

Biii — % of CC3 positive area relative to section area — Control, SN38 (μgml^{-1}): 0.1, 1, 2.5, 5

Ciii — % of BrdU positive area relative to section area — Control, SN38 (μgml^{-1}): 0.1, 1, 2.5, 5