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1 **Prolonged exposure to acetaminophen reduces testosterone production by the human**
2 **fetal testis in a xenograft model**

3

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14

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16

17

18 **One Sentence Summary**

19 Prolonged exposure to therapeutic doses of acetaminophen reduces testosterone production by human fetal testis
20 xenografts.

21

22 **Keywords**

23 Fetal testis, testosterone, acetaminophen, steroidogenesis, xenograft

24

25 **Correspondence and reprint requests**

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29 **Abstract**

30

31 Most common male reproductive disorders are linked to lower testosterone exposure in fetal life, although the
32 factors responsible for suppressing fetal testosterone remain largely unknown. Protracted use of acetaminophen
33 during pregnancy is associated with increased risk of cryptorchidism in sons, but effects on fetal testosterone
34 production have not been demonstrated. We used a validated xenograft model to expose human fetal testes to
35 clinically relevant doses and regimens of acetaminophen. Exposure to a therapeutic dose of acetaminophen for 7
36 days significantly reduced plasma testosterone (45% reduction; $p=0.025$) and seminal vesicle weight (a biomarker
37 of androgen exposure; 18% reduction; $p=0.005$) in castrate host mice bearing human fetal testis xenografts,
38 whereas acetaminophen exposure for just 1 day did not alter either parameter. Plasma acetaminophen
39 concentrations (at 1 hour after the final dose) in exposed host mice were substantially below those reported in
40 humans after a therapeutic oral dose. Subsequent *in utero* exposure studies in rats indicated that the
41 acetaminophen-induced reduction in testosterone likely results from reduced expression of key steroidogenic
42 enzymes (*Cyp11a1*, *Cyp17a1*). Our results suggest that protracted use of acetaminophen (1 week) may suppress
43 fetal testosterone production, which could have adverse consequences. Further studies are required to establish the
44 dose-response and treatment-duration relationships to delineate the maximum dose and treatment period without
45 this adverse effect.

46

47 **Introduction**

48

49 Male reproductive disorders that manifest at birth (cryptorchidism, hypospadias) or in young adulthood (low sperm
50 counts, testicular germ cell cancer) are remarkably common and their incidence may be increasing (1, 2). A major
51 cause of these disorders is believed to be deficiency in testosterone production during a critical period of fetal life
52 (3, 4). However, the factors that might reduce testosterone production or action during fetal life and account for the
53 high prevalence of these disorders are unknown, although it is likely that lifestyle and environmental factors are
54 important (5).

55

56 Acetaminophen is the most commonly used analgesic worldwide, and it is available over the counter in most
57 countries. In the US, more than 65% of pregnant women use acetaminophen at some stage during their pregnancy
58 (6). Maternal use of acetaminophen and subsequent cryptorchidism in male offspring have been linked in several
59 human studies, and experimental investigations in rodents indicate this could be due to reduced testicular hormone
60 production (7-11).

61

62 The aim of the present study was to determine if exposure to a therapeutic dose and regimen of acetaminophen
63 would result in a reduction in testosterone production by the human fetal testis and to identify potential targets in
64 the steroidogenic pathway that may result in reducing testosterone, hence providing a mechanistic link between
65 maternal use of acetaminophen and the occurrence of cryptorchidism and other potential effects in male offspring.
66 Direct identification of the effect of acetaminophen exposure by evaluating testosterone production by the human
67 fetal testis *in situ* is challenging, and use of animal models may not reliably reflect the effects of exposures in
68 humans (12-14). Use of *in vitro* cultures of human fetal testis is feasible, but has limitations and does not always
69 result in the same effects that occur after *in utero* exposure (15). We have thus developed and validated a xenograft
70 model of human fetal testicular development, which reflects physiological development and can be used to test the
71 effects of chemical exposures on testosterone production (12, 16). We used this approach for the present studies.

72

73 **Results**

74

75 **Xenograft survival and host animal health after exposure to acetaminophen**

76 We transplanted a total of 324 fragments of human fetal testis (n=14) tissue into 64 castrated, hCG-treated host
77 mice. Mice were exposed to acetaminophen or vehicle according to three different regimens. Overall graft survival
78 at the end of the experiment was 65%, which is similar to previous studies using this model (12, 16, 17), with no
79 significant differences in graft retrieval rates between treatments or treatment regimens (Table S1). No significant
80 differences in total recovered graft weight were seen between vehicle- and acetaminophen-exposed hosts or
81 between the different treatment regimens (Table S1). Host animals remained healthy, with no significant
82 differences in body weight between vehicle- and acetaminophen-exposed hosts (Table S1). Histological analysis of
83 the xenografts revealed preservation of seminiferous cords and interstitial compartments, with similar appearance
84 between the vehicle- and acetaminophen-exposed xenografts (Fig. 1A).

85

86 **Effect of prolonged exposure of human fetal testis xenografts to high-dose acetaminophen**

87 Our initial experiments investigated the effect of exposure to a single high dose (350 mg/kg) of acetaminophen
88 administered orally daily for 7 days (Fig. 1B), using a regimen previously shown to reduce testosterone production
89 in the rat fetal testis (350 mg/kg, once daily; (9)). We evaluated the effect of this treatment on xenograft
90 testosterone production in two independent ways in the host mice when they were sacrificed 1 hour after the final
91 treatment. We directly measured host plasma testosterone, and we also measured host seminal vesicle (SV) weight,
92 which is a well-established biomarker of androgen exposure in rodents (3). Although treatment with this single
93 daily high dose of acetaminophen did not significantly alter host plasma testosterone concentration 1 hour after the
94 final dose (vehicle controls vs. acetaminophen: 0.35 vs. 0.29 ng/ml; p=0.469; Fig. 1C,D), it did significantly reduce
95 (27% reduction) host seminal vesicle weight (controls vs. acetaminophen: 13.38 vs. 9.75 mg, p=0.0002; Fig. 1E,F),
96 indicating that acetaminophen had reduced overall testosterone production by the xenografts over the duration of
97 the grafting period.

98

99 **Effect of prolonged exposure of human fetal testis xenografts to human-relevant doses of acetaminophen**

100 Because a single daily dose of 350 mg/kg acetaminophen is not human-relevant, we next tested a treatment regimen
101 (20 mg/kg, orally, 3 times daily for 7 days; Fig. 2A) comparable to that recommended for use in humans. Using this
102 regimen, plasma concentrations of acetaminophen (1 hour after the final dose) were $0.74 \pm 0.07 \mu\text{g/ml}$ (Fig. 2B),
103 which is substantially lower than concentrations reported in the serum of normal pregnant women ($20.8 \mu\text{g/ml}$) 0.8
104 hours after a therapeutic dose of acetaminophen (Fig. 2B; (18)). As expected, acetaminophen was undetectable
105 ($<0.1 \mu\text{g/ml}$) in vehicle-exposed host mice (Fig. 2B). Exposure of xenografted mice to this therapeutic dose and
106 regimen of acetaminophen for 7 days resulted in a significant reduction in both host plasma testosterone (45%; 2.49
107 $v. 1.37 \text{ ng/ml}$; $p=0.025$; Fig. 2C,D) and seminal vesicle weight (18%; $7.83 \text{ vs. } 6.42\text{mg}$; $p=0.005$; Fig. 2E,F),
108 compared to vehicle-exposed xenografted controls.

109

110 **Effect of short-term exposure of human fetal testis xenografts to human-relevant doses of acetaminophen**

111 It is assumed that the majority of pregnant women who use acetaminophen do so for a short period of time, so we
112 investigated the effect of a single day's exposure to a therapeutic regimen of acetaminophen (20 mg/kg, orally,
113 three times daily; Fig. 3A), with measurement of plasma testosterone and seminal vesicle weight 1 hour after the
114 final acetaminophen dose. Host mouse plasma acetaminophen concentration was $0.94 \pm 0.27 \mu\text{g/ml}$ in
115 acetaminophen-exposed hosts and was undetectable in vehicle-exposed xenografted controls (Fig. 3B). There was
116 no difference ($p>0.05$) in plasma testosterone (Fig. 3C,D) or seminal vesicle weight (Fig. 3E,F) in acetaminophen-
117 exposed host mice compared to vehicle-exposed controls after this single day of treatment.

118

119 **Effect of acetaminophen exposure on steroidogenesis in the rat fetal testis**

120 Investigation of the mechanisms responsible for the acetaminophen-induced reduction in testosterone production by
121 human fetal testis xenografts is technically challenging due to variation in cellular composition of xenografts which
122 would result in a requirement for a large number of additional fetuses in order to make valid quantitative
123 assessment of steroidogenic enzyme expression. This would be impractical due to limited tissue availability. We
124 therefore used the rat as a model, because we have established the critical period when any reduction in fetal

125 intratesticular testosterone (ITT) can result in a subsequent male reproductive disorder (2, 3). Treatment of pregnant
126 rats with the same therapeutic regimen and dose of acetaminophen as used in the human xenograft studies did not
127 result in any significant suppression of ITT (Fig. S1), so we used the higher dose (350 mg/kg, administered once
128 daily from e13.5; Fig. 4A) because this had been shown to be effective in reducing testosterone production in the
129 fetal rat testis (9). This dose, which resulted in mean blood acetaminophen levels of 44 $\mu\text{g/ml}$, did not adversely
130 affect growth of the mothers or pups, indeed there was evidence for a positive effect of acetaminophen on the
131 weights of both mothers and pups, although the effect on pup weight was not significant if litter bodyweight means
132 were used (Fig. S2). Exposure to acetaminophen significantly suppressed ITT (37% reduction; $p=0.024$) in male
133 pups at e17.5 (the middle of the masculinization programming window, MPW; (3)) 24 hours after the final dose
134 (Fig. 4B), although at 3 hours after dosing the treatment-induced decrease in ITT was not significant ($p=0.098$, Fig.
135 4C). Independent confirmation that this acetaminophen treatment regimen had induced a biologically relevant
136 decrease in ITT was provided by a significant reduction ($p<0.0001$; Fig. 4D) in anogenital index (AGI; calculated
137 by dividing AGD by the cube root of body weight) at e21.5 in acetaminophen-exposed fetuses, because this
138 measure provides a biomarker of fetal androgen exposure during the MPW (3). To investigate the mechanism for
139 acetaminophen-induced reduction in ITT in rat fetuses, we determined the expression of mRNA of key enzymes in
140 the steroidogenic pathway. Expression of *Cyp11a1* ($p=0.013$; Fig. 5A) and *Cyp17a1* ($p=0.025$; Fig. 5B) were both
141 significantly reduced 3 hours after the final dose in acetaminophen-exposed, compared with vehicle-exposed, rat
142 fetal testes, whilst expression of *StAR* and *Hsd3b1* was unchanged (Fig. 5C,D). Expression of *Insl3* and *Sox-9*
143 mRNAs (relevant to testicular descent and Sertoli cells, respectively) were also unchanged in acetaminophen-
144 exposed, compared with vehicle-exposed, rat fetal testes. To determine whether the reduction in *Cyp11a1* and
145 *Cyp17a1* was due to a reduction in the number of Leydig cells, quantification of the Leydig to Sertoli cell ratio
146 (LC:SC) and number of Leydig cells per area (mm^2) was performed on vehicle- and acetaminophen-exposed
147 sections co-stained for 3β -HSD and Sox-9 (Fig. 6A,B). There was no significant difference in LC:SC ($p=0.437$;
148 Fig. 6C) or in LC/mm^3 ($p=0.465$; Fig. 6D) in acetaminophen-exposed compared to vehicle-exposed rat testes.

149

150 **Discussion**

151

152 The present study demonstrates that exposure to acetaminophen in a therapeutic regimen for 7 days reduces
153 testosterone production by the xenografted human fetal testis, an effect that occurs in a context engineered to
154 simulate the normal endocrine environment of the fetal testis (the presence of hCG to stimulate/maintain
155 steroidogenesis) (19). These results have clinical importance for two reasons. First, acetaminophen is used by the
156 majority of pregnant women (6). Second, there is growing evidence that most common male reproductive disorders,
157 which can affect up to 1 in 6 males, may be attributed to suboptimal androgen exposure during fetal life (4), a
158 parameter which we presently show can be affected by exposure of the fetal human testis to therapeutically-relevant
159 doses of acetaminophen. This is independently supported by several epidemiological studies, which have shown
160 that protracted maternal use of acetaminophen is associated with increased risk of cryptorchidism in the exposed
161 offspring (7-11), and reduced fetal androgen exposure is an established cause of cryptorchidism (3, 4). Moreover, it
162 has recently been shown that deficiency in fetal testosterone might also result in compromised adult testosterone
163 production, which has potential implications for more general health consequences (20).

164

165 In the present study, exposure to a high dose (350 mg/kg, once daily) of acetaminophen for 7 days resulted in a
166 reduction in weight of the androgen-dependent seminal vesicles in host mice, indicating a reduction in testosterone
167 production by the human fetal testis xenografts over the duration of the exposure. However, the plasma testosterone
168 measured 1 hour after the final dose showed a non-significant reduction. This may be related to the single daily
169 dosing schedule. This concept is supported by two further findings from the present study. First, in rats exposed *in*
170 *utero* to an equivalent high dose regimen administered once daily, the reduction in ITT had not occurred by 3 hours
171 after the final dose, although there was a reduction when ITT was measured 24 hours after the final dose. Second,
172 we demonstrated a reduction (measured 1 hour after the final dose) in testosterone in xenografted host mice after a
173 three times daily (for 7 days) therapeutic dosing regimen of a substantially lower acetaminophen dose.

174

175 The present studies show that a therapeutic acetaminophen regimen (20 mg/kg, three times daily) for 7 days results
176 in a 45% reduction in testosterone production by human fetal testis xenografts, with a reduction (18%) in SV
177 weight in host animals. This reduction was evident despite the concomitant presence of physiological stimulation of
178 testosterone production by hCG (to mimic the human *in utero* environment), administered to the host mice, which
179 has been shown to increase testosterone production by human fetal testis xenografts (16). However, exposure to the
180 same acetaminophen dosing regimen for a single day did not result in a reduction in testosterone or SV weight. This
181 suggests, at least in this model system, that it is only protracted acetaminophen exposure that suppresses
182 testosterone production.

183

184 To confirm the potential human health relevance of our findings, we measured plasma acetaminophen
185 concentrations in host mice, and found concentrations of 0.74-0.94 $\mu\text{g/ml}$ at 1 hour after a dose (therapeutic
186 regimen). This is well below the concentrations reported in adult humans (6 $\mu\text{g/ml}$) at the same time-point (1 hour)
187 after the equivalent oral dose, and using the same type of assay (LC/MS) as the present study (21). In addition, a
188 study that investigated pregnant and non-pregnant women demonstrated a maximum serum concentration at 0.8 h
189 after a dose of 20.8 $\mu\text{g/ml}$ during pregnancy and 23.7 $\mu\text{g/ml}$ in the non-pregnant state (18). The reason for the
190 difference between the acetaminophen concentrations described in the two previous studies is unclear but may
191 relate, at least in part, to the type of assay used. Acetaminophen passes readily across the placenta, with no
192 difference in blood concentrations between mother and fetus after an oral therapeutic dose (1g) (22), thus our
193 results suggest that the testis xenografts in our studies were exposed to concentrations of acetaminophen that are not
194 higher, and indeed substantially lower, than those likely to occur in the human fetus after maternal use of a standard
195 therapeutic dose. Previous *in vitro* studies have shown that acetaminophen can reduce testosterone production in the
196 rat fetal testis, demonstrating a direct effect not mediated by metabolites (9). These considerations indicate that the
197 present findings might therefore underestimate the effects of acetaminophen exposure in humans, if the effect of
198 acetaminophen on testosterone production by xenografts is dose-dependent. In this regard, it also appears that the
199 human fetal testis may be more sensitive to the adverse steroidogenic effects of acetaminophen than the fetal rat

200 testis, as evidenced by our failure to detect any significant steroidogenic effect of a therapeutic dosing regimen in
201 rats.

202

203 A limitation of our study is that the xenograft model may not accurately reflect the *in utero* situation for humans.
204 However, it is not possible to test directly the effects of acetaminophen on fetal testis testosterone production in
205 pregnant women. Our xenograft system has been shown to model normal human fetal testis development, including
206 its steroidogenic function (16, 23), and previous studies have demonstrated its validity as a model to assess the
207 effects of potential endocrine disruptors on testosterone production. These studies have shown consistency of
208 results in the xenograft system with those of *in vitro* studies in the human fetal testis and *in vivo* studies in non-
209 human primates (12, 13, 15, 24, 25) . This includes studies that utilise exposures to known inhibitors of
210 steroidogenesis (23), or fetal testis tissue xenografts from species where exposure is known to reduce testosterone
211 *in vivo* (12, 13), as positive controls. Therefore we consider the xenograft model to be a dynamic and reliable model
212 with which to investigate the effects of chemical and drug exposures on steroidogenesis (12). Our present results
213 contrast with a study that investigated the effects of acetaminophen exposure on first trimester (8-12 weeks
214 gestation) human fetal testes *in vitro*, and found no effect on testosterone production after 24-72 hours of culture
215 (26). This discrepancy could indicate differential effects of acetaminophen exposure in first trimester compared
216 with the second trimester human fetal testes used in this study. However, it is perhaps more likely that the lack of
217 effect of acetaminophen on testosterone production *in vitro* may relate to inconsistent effects on steroidogenesis
218 that have been shown to occur when compared to the *in utero* response (15), and these differential effects may be
219 dependent on the specific culture conditions (27).

220

221 To provide mechanistic support for our findings in an *in utero* model, we exposed pregnant rats to acetaminophen
222 using two different regimens. We demonstrated that once daily exposure to acetaminophen from e13.5 resulted in a
223 reduction in ITT 24 hours after the final dose at e17.5. This was associated with a reduction in AGI at e21.5, which
224 is an established indicator of fetal androgen exposure (4). Similar effects on AGI after acetaminophen exposure in
225 fetal rats have been described previously (9). Having demonstrated a reduction in testosterone production, we

226 investigated steroidogenic enzyme expression in the rat fetal testis after exposure to acetaminophen. We showed a
227 significant reduction in both *Cyp11a1* and *Cyp17a1* mRNA expression 3 hours after the final dose of
228 acetaminophen. *Cyp11a1* catalyzes the conversion of cholesterol to pregnenolone in the gonad and is the rate-
229 limiting enzyme in the steroidogenic pathway (19, 28). We have previously shown in fetal rats that a reduction in
230 *Cyp11a1* mRNA of similar magnitude to that in the present study also results in a reduction in *Cyp11a1* protein
231 expression (29). *Cyp17a1* is responsible for catalyzing the conversion of 17-hydroxy-pregnenolone to
232 dehydroepiandrosterone and is also an important determinant of testosterone production that is subject to regulation
233 and perturbation (30). To confirm that the reduction in expression of these key steroidogenic enzymes was not due
234 to a loss of Leydig cells, we showed that mRNA expression of several other Leydig cell products, including 3 β -
235 HSD and *Insl3*, was unchanged by exposure to acetaminophen. Furthermore, we took advantage of the stable
236 mRNA expression of *Hsd3b1* (Leydig cell) and *Sox-9* (Sertoli cell) to perform Leydig cell counts, which also
237 indicated that LC number was unchanged after exposure to acetaminophen, determined by two independent
238 methods of quantification. Whether the effects of acetaminophen on expression of *CYP11a1* and *CYP17a1* are
239 direct or indirect remains to be established. However, these results provide mechanistic support for our findings of
240 reduced testosterone production by the xenografted human fetal testis after exposure to acetaminophen.

241

242 The present findings provide the beginnings of a mechanistic explanation for the association between protracted
243 acetaminophen use in human pregnancy and the increased occurrence of cryptorchidism in sons (9, 11), because the
244 majority of cases of cryptorchidism involve failure of the androgen-dependent phase of testis descent (31, 32). Our
245 findings might also have relevance in terms of other associated male reproductive disorders, such as hypospadias,
246 testicular germ cell cancer, and low sperm counts, which are also linked to reduced androgen exposure *in utero* (4).

247

248 In conclusion, we show that 1 week's exposure to a human-equivalent therapeutic regimen of acetaminophen
249 results in reduced testosterone production by xenografted human fetal testis tissue, whilst short-term (1 day) use
250 does not result in any long-lasting suppression of testosterone production. Because our results are based on the use
251 of a model system, it is not possible to translate our findings into a categorical recommendation regarding what

252 would be safe or unsafe use of acetaminophen by women during pregnancy. However, a pragmatic approach may
253 involve the avoidance of protracted use of acetaminophen during pregnancy where possible, a suggestion that is
254 underscored by the epidemiological data linking protracted acetaminophen use in pregnancy with increased risk of
255 cryptorchidism in sons (7-9, 11).

256

257 **Materials and Methods**

258

259 **Experimental design**

260 Given the reported association between maternal use of acetaminophen and cryptorchidism, we aimed to
261 determine whether exposure to acetaminophen reduces testosterone production by the human fetal testis. We
262 performed a controlled laboratory experiment that used a validated xenograft system to expose human fetal testis
263 tissue to ‘human-equivalent’ therapeutic doses of acetaminophen administered orally to the immunocompromised
264 host mice. In addition, studies were also performed in rats to determine the mechanism for acetaminophen’s
265 effects on fetal testis steroidogenesis. Inclusion criteria and measured endpoints were defined before the start of
266 the study. For all experiments, the sample sizes for human fetal testes (minimum n=4 for each treatment regimen)
267 and for rat fetuses (n=3 litters per group) were based on those required to achieve statistical significance in
268 previous studies using the same methodology (12, 17). The study was stopped once the required number of
269 experiments had been conducted, and data were analyzed after the cessation of the study. No outliers were
270 excluded. To compare the effects of treatment versus vehicle for each individual human fetal testis, we grafted
271 tissue from each fetus into 3-6 replicate host mice and randomly allocated these to receive either acetaminophen or
272 vehicle treatment. The endpoints included measurement of seminal vesicle (SV) weight and plasma testosterone in
273 host mice bearing testis tissue xenografts. Seminal vesicles were weighed by a single investigator and verified by
274 a second investigator who was blinded to the treatment group. Blood was taken from host mice, and plasma was
275 extracted for analysis of testosterone and acetaminophen using validated assays with 3 replicates per sample. One
276 host mouse was excluded from analysis because of incomplete castration. For the rat studies, pregnant dams were
277 randomly allocated to receive either acetaminophen or vehicle via oral administration. The investigators
278 performing the blood sample, ITT, and steroidogenic enzyme analysis were blinded to the treatment group.

279

280 **Ethics statement**

281 Human fetal testes were obtained after elective termination of pregnancy, according to the Declaration of Helsinki
282 - Ethical Principles for Medical Research Involving Human Subjects. Ethical approval for the study was obtained

283 from the South-East Scotland Research Ethics Committee (Reference number - LREC08/S1101/1). Women gave
284 written informed consent. Animal studies received specific approval by the UK Home Office, including ethical
285 approval, and were performed according to the Animal (Scientific Procedures) Act 1986.

286

287 **Human xenografting studies**

288 **Animals**

289 For xenografting studies, male CD1 nude (host) mice (aged 4-6 weeks; n=64; Charles River UK) were
290 anaesthetised by inhalation of isoflurane and castrated through a scrotal incision. Castration was performed at least
291 2 weeks before xenografting. After castration, mice received analgesia (Carprofen; Pfizer) in the drinking water
292 for 3 days.

293

294 **Human tissue and xenografting procedure**

295 Human fetal testes (n=14; 14 weeks n=3, 15 weeks n=6, 16 weeks n=1, 17 weeks n=3, 20 weeks n=1) were grafted
296 into castrate host mice as previously described (16). Briefly, a small portion of each testis was immediately fixed as
297 a pre-graft control, whilst the remainder was placed immediately into ice-cold medium containing Liebowitz L-15
298 with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non-essential amino acids (all Sigma
299 Aldrich) for xenografting. Small pieces (1 mm³ approx.) of testis tissue were inserted subcutaneously under the
300 dorsal skin of the mice using a 13G cancer implant needle (Popper and Sons). Grafts (4-6 per mouse) were inserted
301 on either side of the midline. In general, 3-6 mice were xenografted with tissue from each fetus, and mice were
302 maintained for 7 days to allow grafts to establish a blood supply before any host treatments commenced.

303

304 **Treatments**

305 One week after grafting, host mice commenced treatment with subcutaneous injection of human chorionic
306 gonadotropin (20 IU hCG every 72 hours; Pregnyl, Organon Laboratories) to mimic the human *in utero*
307 environment (16). Host mice were also randomly allocated to receive either acetaminophen or vehicle (corn oil) by
308 daily oral administration according to one of the following regimens:

309

- 310 1) High dose 7 day - 350 mg/kg, once daily for 7 days with analysis 1 hour after the final dose
- 311 2) Therapeutic dose 7 day - 20 mg/kg, three times daily for 7 days with analysis 1 hour after the final dose
- 312 3) Therapeutic dose 1 day - 20 mg/kg, three times daily for 1 day with analysis 1 hour after the final dose

313

314 **Xenograft retrieval**

315 Host mice were sacrificed by cervical dislocation, and blood was obtained by cardiac puncture for assessment of
316 plasma testosterone and acetaminophen. Testosterone production and action was assessed by measuring plasma
317 testosterone and seminal vesicle weight (16). Xenografts were retrieved and weighed prior to fixation in Bouins
318 Fluid (Clin-Tech). Fixed sections were stained with Hematoxylin and Eosin (H+E).

319

320 **Testosterone assay**

321 Plasma testosterone levels in xenografted hosts were measured at termination by competitive radioimmunoassay
322 using an extraction-based in-house radioimmunoassay method described previously (33). Testosterone levels were
323 expressed as ng/ml (human xenografts). All samples were analysed in a single assay with 3 replicates. The
324 detection limit was 45 pg/ml and the intra-assay CV 8%.

325

326 **Acetaminophen assay**

327 Acetaminophen (APAP) was extracted from plasma by liquid-liquid extraction with acidified HPLC-grade
328 methanol (Fisher Scientific). Briefly, 10 μ L plasma was enriched with 10 ng deuterium-labelled acetaminophen
329 (APAP-d₄; Santa Cruz Biotechnology Inc) as internal standard, 0.8 mL methanol (w/ 0.2% acetic acid, Sigma
330 Aldrich) was added, and the samples were then vortexed and incubated for 20 min on ice. After centrifugation

331 (3000 g, 10 min, 10°C), the supernatant was reduced to dryness under nitrogen at 40°C, reconstituted in mobile
332 phase (200 µL water/methanol (65:35, v/v), and centrifuged for a second time.

333

334 Chromatographic separation was achieved using an Aria CTC autosampler and Allegros pump on an ACE Excel 2
335 SuperC18 column (150 x 3 mm; 2 µm) protected by a Kinetex KrudKatcher (Phenomenex) at 20°C, and
336 acetaminophen was detected on a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Fisher
337 Scientific). The mobile phase consisted of 0.1% formic acid (Sigma Aldrich) in water and 0.1% formic acid in
338 methanol (B) at a flow rate of 0.3 mL/min. Gradient elution was achieved with a total run time of 9 min from 35%
339 to 5% B. APAP eluted at 3.95 mins. The mass spectrometer was operated in positive ion electrospray mode
340 (300°C, 3 kV). Transitions monitored were m/z 152 → 110 and m/z 156.1 → 114.1 for APAP and APAP-d4,
341 respectively.

342

343 **Rat *in utero* exposure studies**

344 **Animals and treatments**

345 Wistar rats were maintained according to UK Home Office guidelines and were fed a soy-free breeding diet
346 (RM3(E) soya free; SDS). Housing conditions were carefully controlled (lights on at 0700, off at 1900 h,
347 temperature 19–21°C, GOLD shavings and LITASPEN standard bedding (SPPS). Time-mated female rats were
348 subjected to once daily treatment with either acetaminophen (high dose - 350 mg/kg/d; therapeutic dose – 20
349 mg/kg three times daily, in corn oil) or vehicle (corn oil) by oral gavage commencing at e13.5, with measurement
350 of ITT at e17.5 (3 hours or 24 hours after the final dose) and AGI at e21.5.

351

352 To acquire fetal samples, rat dams were sacrificed by inhalation of CO₂ followed by cervical dislocation. Fetuses
353 were removed, decapitated, and placed in ice-cold phosphate buffered solution (PBS; Sigma-Aldrich). Fetal
354 anogenital distance (AGD) was measured in males between the base of the phallus and the anterior margin of the
355 anus using digital calipers (Faithfull Tools). AGI was calculated by dividing AGD by the cube root of body
356 weight. From each fetus, one testis was microdissected, snap frozen, and stored at -70°C for determination of ITT

357 or for gene expression analysis. For ITT, testis tissue was placed in tubes containing Phosphate Buffered Saline
358 (PBS) and a metal bead (Qiagen) and homogenized using two cycles with the tissue lyser (Qiagen). Total
359 testosterone content of the lysate was measured using a validated radioimmunoassay as described above. The
360 remaining testis was immediately placed in Bouins fluid for 1 hour followed by transfer to 70% ethanol. Fixed
361 testes were embedded in paraffin using standard processes, and sections of 5 μm thickness were prepared for
362 subsequent immunofluorescence.

363

364 **Double immunofluorescence for Sox-9 and 3 β -HSD in fetal rat testis**

365 In order to facilitate analysis of different cell populations, specific antibodies were used for co-
366 immunolocalization of the Sertoli cell marker Sox-9 and the Leydig cell marker 3 β -HSD. Sections (5 μm) were
367 dewaxed in xylene and rehydrated in a graded ethanol series, followed by incubation for 30 min at room
368 temperature (RT) in 3% (v/v) H₂O₂ in methanol in order to block endogenous peroxidase. Sections were then
369 washed twice (5 min each) in TBS. All subsequent incubations were carried out in a humidity box (Thermo Fisher
370 Scientific) at RT unless otherwise stated, and washes between incubation steps were in TBS (2 x 5 min). To block
371 non-specific binding, sections were incubated for 30 min in normal chicken serum (NCS; Biosera) diluted 1:5 in
372 TBS containing 5% (w/v) Bovine Serum Albumin (NCS/TBS/BSA), followed by incubation overnight at 4°C
373 with anti-Sox-9 antibody (Merck Millipore) diluted 1:6000 in NCS/TBS/BSA. Sections were then incubated for
374 30 minutes with peroxidase-conjugated chicken anti-rabbit secondary antibody (DAKO), diluted 1:200 in
375 NCS/TBS/BSA, followed by incubation for 10 min with tyramide (TSA-Plus Cyanine3 System; Perkin Elmer Life
376 Sciences) according to the manufacturer's instructions. Sections were then subjected to antigen retrieval by boiling
377 in a pressure cooker in 0.01 M citrate buffer (pH 6.0) for 5 min and left to cool for 20 min, followed by blocking
378 for 30 min in NCS/TBS/BSA and overnight incubation at 4°C with anti-3 β -HSD antibody (Santa Cruz
379 Biotechnology) diluted 1:200 in NCS/TBS/BSA. Sections were then incubated for 30 min with peroxidase-
380 conjugated chicken anti-goat secondary antibody (Santa Cruz Biotechnology) diluted 1:200 in NCS/TBS/BSA,
381 followed by incubation for 10 min with tyramide (TSA-Plus Cyanine5 System; Perkin Elmer Life Sciences).

382 Sections were mounted with Permafluor (Thermo Fisher Scientific). Fluorescent images of complete testis cross-
383 sections were captured using an LSM 710 Axio Observer Z1 confocal laser microscope (Carl Zeiss Ltd.).

384

385 **Quantification of Leydig cells**

386 For quantification of Leydig cells in vehicle- and acetaminophen-exposed testes, the numbers of both Sertoli cells
387 (SC; Sox-9-positive nuclei), as an internal reference control, and Leydig cells (LC; nuclei associated with 3 β -
388 HSD-positive cytoplasm) were quantified using two independent measures, namely, LC:SC and total LC/mm².
389 We used images of complete fetal rat testes cross-sections (n=4; 2 sections per animal) immunostained for Sox-9
390 and 3 β -HSD as described above. Fluorescent images were examined with ZEN Lite software (Carl Zeiss Ltd), and
391 the contour tool was used to draw around the testis margin and automatically calculate the cross-section area
392 (mm²). The grid overlay tool was used to facilitate systematic scanning of sections and cell counting. Image-Pro
393 6.2 with Stereologer plug-in software (MagWorldwide) was used to manually tag/count cells. All SC and LC
394 nuclei within the sections were counted. For both methods of LC quantification, cell counts from two replicate
395 sections per fetal testis were aggregated to give total SC and LC counts per animal (1405 +/- 343.2 cells).

396

397 **Gene expression analysis**

398 For quantitative analysis of gene expression by RT-PCR, total RNA was extracted from vehicle- and
399 acetaminophen-exposed rat fetal testes (n=11-12) using the RNeasy Micro Kit with on-column DNase digestion
400 (Qiagen). Random hexamer primed cDNA was prepared using the Applied Biosystems Taqman RT kit (Applied
401 Biosystems). Quantitative real time PCR (qRT-PCR) was performed on the ABI Prism Sequence Detection
402 System (Applied Biosystems). Expression of rat *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Insl3*, and *Sox-9* RNA was
403 determined using the Roche Universal Probe Library (*Star* forward primer: 5'-TCACGTGGCTGCTCAGTATT-
404 3', reverse primer: 5'-GGGTCTGTGATAAGACTTGGTTG-3', probe number 83 Cat no. 04689062001; *Cyp11a1*
405 forward primer: 5'-TATTCCGCTTTGCCTTTGAG-3', reverse primer 5'-CACGATCTCCTCCAACATCC-3',
406 probe number 9 Cat no. 04685075001; *Cyp17a1* forward primer: 5'-CATCCCCACAAGGCTAAC-3', reverse
407 primer: 5'-TGTGTCCTTGGGGACAGTAAA-3', probe number 67 Cat no. 04688660001; *Hsd3b1* forward

408 primer: 5'-GACCAGAAACCAAGGAGGAA-3', reverse primer: 5'-CTGGCACGCTCTCCTCAG-3', probe
409 number 105 Cat no. 04692241001); *Ins13* forward primer: 5'-TTC CTC ACC AGG CTT CTC AG-3', reverse
410 primer: 5'-CAG ACC CAA AAG GTC TTG CT-3', probe number 71 Cat. No. 04688945001; *Sox9* forward
411 primer: 5'-ATC TTC AAG GCG CTG CAA-3', reverse primer: 5'-CGG TGG ACC CTG AGA TTG-3', probe
412 number 63 Cat. No. 04688627001; (Roche Applied Sciences). The expression of each gene was normalized using
413 a ribosomal 18S internal control (Applied Biosystems Cat no. 4308329). All samples were run in triplicate and
414 compared to adult testis control cDNA (Ambion).

415

416 **Statistical Analysis**

417 For human xenografting studies, results were analysed by two-way ANOVA to account for inter-individual
418 variation between fetuses, as previously described (12). For each treatment regimen, tissue from each fetus (n=4-5)
419 was considered an individual experiment. Tissue from each fetus was grafted into 3-6 replicate host mice (4-6 grafts
420 per mouse), and each mouse was randomly allocated to receive vehicle or acetaminophen. For rat studies, analysis
421 was performed using unpaired t-tests. $P < 0.05$ was used to determine significance. No outliers were excluded.

422 **Supplementary Materials**

423

424 Table S1 - Xenograft retrieval rates, total graft weight and host mouse body weight following exposure to
425 acetaminophen

426

427 Figure S1 - Low-dose acetaminophen exposure and intratesticular testosterone in the fetal rat

428

429 Figure S2 – Effect of exposure to acetaminophen on pregnant rats (bodyweight) and their male offspring
430 (bodyweight/AGD)

431

432 **References and notes**

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- 533

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545

546 **Role of the authors**

547 Conceived and designed the experiments: RTM, RMS

548 Performed the experiments: RTM, JM, SVD, ZJ, NH, TC, M C-M, AJ, CM

549 Analyzed the data: RTM, LBS, AD, RAA, RMS, SVD, CM

550 Contributed reagents/materials/analysis tools: RTM, LBS, RAA

551 Wrote the paper: RTM, LBS, RMS

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553

554 **Competing interests**

555 The authors declare that they have no competing interests.

556

557 **Figure Legends**

558

559 **Figure 1. High-dose acetaminophen exposure and steroidogenesis by human fetal testis xenografts**

560 A) Histological appearance (H+E staining) of xenografts exposed to vehicle (VEH) and high-dose acetaminophen
561 (ACET) with corresponding low-power images of whole grafts (inset). Seminiferous cord structure is maintained
562 during the grafting period. Scale bar – 50 μ m. B) High-dose (350 mg/kg, once daily for 7 days) acetaminophen
563 exposure regimen for human fetal testis xenografts.. Plasma testosterone (C, D) and seminal vesicle weight (E, F)
564 in host mice (n=12-13 host mice) bearing human fetal (n=5 total; 15 weeks n=3, 16 weeks n=1, 17 weeks n=1)
565 testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Panels D and F show
566 overall mean data. Graphs show means +/- SEM. Data analyzed by two-way ANOVA.

567

568 **Figure 2. Therapeutic acetaminophen exposure and steroidogenesis by human fetal testis xenografts**

569 A) Therapeutic dose (20 mg/kg, three times daily for 7 days) acetaminophen exposure regimen for human fetal
570 testis xenografts. Plasma acetaminophen (B) and testosterone (C, D) concentrations and seminal vesicle weight (E,
571 F) in host mice (n=11-12 host mice) bearing human fetal (n=5 total; 14 weeks n=3, 15 weeks n=1, 17 weeks n=1)
572 testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Dashed line in B shows
573 the mean acetaminophen concentration in humans exposed to an equivalent dose of acetaminophen (21). Panels D
574 and F show overall mean data. Graphs show means +/- SEM. Data analyzed by two-way ANOVA.

575

576 **Figure 3. Short-term therapeutic acetaminophen and steroidogenesis by human fetal testis xenografts**

577 A) Therapeutic dose (20 mg/kg, three times daily for 1 day) acetaminophen exposure regimen for human fetal
578 testis xenografts. Plasma acetaminophen (B) and testosterone (C, D) concentrations and seminal vesicle weight (E,
579 F) in host mice (n=8 host mice) bearing human fetal (n=4 total; 15 weeks n=2, 17 weeks n=1, 20 weeks n=1)
580 testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Dashed line in B shows
581 the mean acetaminophen concentration in humans exposed to an equivalent dose of acetaminophen (21). Panels D
582 and F show overall mean data. Graphs show mean +/- SEM. Data analyzed by two-way ANOVA.

583

584 Figure 4. High-dose acetaminophen exposure and steroidogenesis by the fetal rat testis

585 A) Schematic for acetaminophen dosing regimens used in pregnant rats. Acetaminophen treatment (350 mg/kg
586 once daily) commenced at e13.5 and litters were taken at e17.5 for intratesticular testosterone (ITT) measurements
587 at 24 h (B; n=23-25) and 3 h (C; n=20-24) after the final treatment dose. Anogenital index (AGI), a biomarker of
588 androgen exposure, was measured at e21.5 (D; n=39-45), after daily treatment from e13.5 with acetaminophen
589 (ACET; red) or vehicle (VEH; blue). Graphs show mean +/- SEM. Data analyzed by unpaired t-test.

590

591 Figure 5. High-dose acetaminophen exposure and mRNA expression in the rat fetal testis

592 Treatments commenced at e13.5 and tissue was collected at e17.5, 3 h after the final treatment. Results show
593 mRNA expression relative to human adult testis cDNA for A) *Cyp11a1*, B) *Cyp17a1*, C) *Hsd3b1*, D) *StAR*, E) *Insl3*
594 and F) *Sox9* after acetaminophen (ACET; red) or vehicle (VEH; blue) exposure (n=11-12). Graphs show mean +/-
595 SEM. Data analyzed by unpaired t-test.

596

597 Figure 6. High-dose acetaminophen exposure and Leydig cell number in the rat fetal testis

598 Treatments commenced at e13.5 and tissue was collected at e17.5, 3 h after the final treatment. A) Double
599 immunofluorescence for 3 β -HSD (Leydig cells; green) and Sox-9 (Sertoli cells; red) in vehicle- (VEH; A) and
600 acetaminophen- (ACET; B) exposed rat fetal testes. Scale bar – 50 μ m. C) Quantification of Leydig cell to Sertoli
601 cell ratio (LC:SC) and D) Leydig cells/mm² for acetaminophen (ACET; red) or vehicle (VEH; blue) exposure
602 (n=4). Graphs show mean +/- SEM. Data analyzed by unpaired t-test.