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One Sentence Summary

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

Keywords

Fetal testis, testosterone, acetaminophen, steroidogenesis, xenograft

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Abstract

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

Introduction

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

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

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

Results

Xenograft survival and host animal health after exposure to acetaminophen

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

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

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

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

Because a single daily dose of 350 mg/kg acetaminophen is not human-relevant, we next tested a treatment regimen (20 mg/kg, orally, 3 times daily for 7 days; Fig. 2A) comparable to that recommended for use in humans. Using this regimen, plasma concentrations of acetaminophen (1 hour after the final dose) were 0·74 ± 0·07 μg/ml (Fig. 2B), which is substantially lower than concentrations reported in the serum of normal pregnant women (20.8 μg/ml) 0.8 hours after a therapeutic dose of acetaminophen (Fig. 2B; (18)). As expected, acetaminophen was undetectable (<0·1 μg/ml) in vehicle-exposed host mice (Fig. 2B). Exposure of xenografted mice to this therapeutic dose and regimen of acetaminophen for 7 days resulted in a significant reduction in both host plasma testosterone (45%; 2.49 v. 1.37 ng/ml; p=0·025; Fig. 2C,D) and seminal vesicle weight (18%; 7.83 vs. 6.42mg; p=0·005; Fig. 2E,F), compared to vehicle-exposed xenografted controls.

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

It is assumed that the majority of pregnant women who use acetaminophen do so for a short period of time, so we investigated the effect of a single day's exposure to a therapeutic regimen of acetaminophen (20 mg/kg, orally, three times daily; Fig. 3A), with measurement of plasma testosterone and seminal vesicle weight 1 hour after the final acetaminophen dose. Host mouse plasma acetaminophen concentration was 0·94 ± 0·27 μg/ml in acetaminophen-exposed hosts and was undetectable in vehicle-exposed xenografted controls (Fig. 3B). There was no difference (p>0·05) in plasma testosterone (Fig. 3C,D) or seminal vesicle weight (Fig. 3E,F) in acetaminophen-exposed host mice compared to vehicle-exposed controls after this single day of treatment.

Effect of acetaminophen exposure on steroidogenesis in the rat fetal testis

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

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

Discussion

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

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

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

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

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

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

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

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

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

In conclusion, we show that 1 week's exposure to a human-equivalent therapeutic regimen of acetaminophen results in reduced testosterone production by xenografted human fetal testis tissue, whilst short-term (1 day) use does not result in any long-lasting suppression of testosterone production. Because our results are based on the use 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

Materials and Methods

Experimental design

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

Ethics statement

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

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

Human xenografting studies

Animals

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

Human tissue and xenografting procedure

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

Treatments

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

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

Xenograft retrieval

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

Testosterone assay

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

Acetaminophen assay

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

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

Chromatographic separation was achieved using an Aria CTC autosampler and Allegros pump on an ACE Excel 2 SuperC18 column (150 x 3 mm; 2 µm) protected by a Kinetex KrudKatcher (Phenomenex) at 20°C, and acetaminophen was detected on a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Fisher Scientific). The mobile phase consisted of 0.1% formic acid (Sigma Aldrich) in water and 0.1% formic acid in 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% to 5% B. APAP eluted at 3.95 mins. The mass spectrometer was operated in positive ion electrospray mode (300°C, 3 kV). Transitions monitored were *m/z* 152 → 110 and *m/z* 156.1 → 114.1 for APAP and APAP-d4, respectively.

Rat *in utero* exposure studies

Animals and treatments

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

To acquire fetal samples, rat dams were sacrificed by inhalation of CO_2 followed by cervical dislocation. Fetuses were removed, decapitated, and placed in ice-cold phosphate buffered solution (PBS; Sigma-Aldrich). Fetal anogenital distance (AGD) was measured in males between the base of the phallus and the anterior margin of the anus using digital calipers (Faithfull Tools). AGI was calculated by dividing AGD by the cube root of body 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 3β-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 3β-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) H_2O_2 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-3β-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).

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

Quantification of Leydig cells

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

Gene expression analysis

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

primer: 5'-GACCAGAAACCAAGGAGGAA-3', reverse primer: 5'-CTGGCACGCTCTCCTCAG-3', probe number 105 Cat no. 04692241001); *Insl3* forward primer: 5'-TTC CTC ACC AGG CTT CTC AG-3', reverse primer: 5'-CAG ACC CAA AAG GTC TTG CT-3', probe number 71 Cat. No. 04688945001; *Sox9* forward primer: 5'-ATC TTC AAG GCG CTG CAA-3', reverse primer: 5'-CGG TGG ACC CTG AGA TTG-3', probe number 63 Cat. No. 04688627001; (Roche Applied Sciences). The expression of each gene was normalized using a ribosomal 18S internal control (Applied Biosystems Cat no. 4308329). All samples were run in triplicate and compared to adult testis control cDNA (Ambion).

Statistical Analysis

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

Supplementary Materials

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

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

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

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Competing interests

The authors declare that they have no competing interests.

Figure Legends

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

A) Histological appearance (H+E staining) of xenografts exposed to vehicle (VEH) and high-dose acetaminophen (ACET) with corresponding low-power images of whole grafts (inset). Seminiferous cord structure is maintained during the grafting period. Scale bar – 50 μm. B) High-dose (350 mg/kg, once daily for 7 days) acetaminophen exposure regimen for human fetal testis xenografts.. Plasma testosterone (C, D) and seminal vesicle weight (E, F) 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) testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Panels D and F show overall mean data. Graphs show means +/- SEM. Data analyzed by two-way ANOVA.

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

A) Therapeutic dose (20 mg/kg, three times daily for 7 days) acetaminophen exposure regimen for human fetal testis xenografts. Plasma acetaminophen (B) and testosterone (C, D) concentrations and seminal vesicle weight (E, 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) testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Dashed line in B shows the mean acetaminophen concentration in humans exposed to an equivalent dose of acetaminophen (21). Panels D and F show overall mean data. Graphs show means +/- SEM. Data analyzed by two-way ANOVA.

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

A) Therapeutic dose (20 mg/kg, three times daily for 1 day) acetaminophen exposure regimen for human fetal testis xenografts. Plasma acetaminophen (B) and testosterone (C, D) concentrations and seminal vesicle weight (E, 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) testis xenografts after exposure to acetaminophen (red; ACET) or vehicle (blue; VEH). Dashed line in B shows the mean acetaminophen concentration in humans exposed to an equivalent dose of acetaminophen (21). Panels D 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 3β-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 μm. C) Quantification of Leydig cell to Sertoli
601 cell ratio (LC:SC) and D) Leydig cells/mm^2 for acetaminophen (ACET; red) or vehicle (VEH; blue) exposure
602 (n=4). Graphs show mean +/- SEM. Data analyzed by unpaired t-test.