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Moïra Rossitto, Candice Marchive, Alain Pruvost, Eli Sellem, Aurelie Ghettsas, et al.. Intergenerational effects on mouse sperm quality after in utero exposure to acetaminophen and ibuprofen. *FASEB Journal*, 2019, 33 (1), pp.339-357. 10.1096/fj.201800488RRR . hal-01832367

HAL Id: hal-01832367

<https://hal.umontpellier.fr/hal-01832367>

Submitted on 22 Jun 2020

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Intergenerational effects on mouse sperm quality after *in utero* exposure to acetaminophen and ibuprofen

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ABSTRACT: Nonsteroidal antiinflammatory drugs and analgesic drugs, such as *N*-acetyl-*p*-aminophenol (APAP; acetaminophen, paracetamol), are widely used by pregnant women. Accumulating evidence has indicated that these molecules can favor genital malformations in newborn boys and reproductive disorders in adults. However, the consequences on postnatal testis development and adult reproductive health after exposure during early embryogenesis are still unknown. Using the mouse model, we show that *in utero* exposure to therapeutic doses of the widely used APAP-ibuprofen combination during the sex determination period leads to early differentiation and decreased proliferation of male embryonic germ cells, and early 5-methylcytosine and extracellular matrix protein deposition in 13.5 d postcoitum exposed testes. Consequently, in postnatal testes, Sertoli-cell maturation is delayed, the Leydig-cell compartment is hyperplastic, and the spermatogonia A pool is decreased. This results in a reduced production of testosterone and in epididymal sperm parameter defects. We observed a reduced sperm count (19%) *in utero*-exposed (F0) adult males and also a reduced sperm motility (40%) in their offspring (F1) when both parents were exposed, which leads to subfertility among the 6 mo old F1 animals. Our study suggests that the use of these drugs during the critical period of sex determination affects the germ-line development and leads to adverse effects that could be passed to the offspring.—Rossitto, M., Marchive, C., Pruvost, A., Sellem, E., Ghetas, A., Badiou, S., Sutra, T., Poulat, F., Philibert, P., Boizet-Bonhoure, B. Intergenerational effects on mouse sperm quality after *in utero* exposure to acetaminophen and ibuprofen.

KEY WORDS: NSAID · analgesic · germ cell · differentiation · sperm parameters

ABBREVIATIONS: ALC, adult Leydig cell; APAP, *N*-acetyl-*p*-aminophenol (acetaminophen, paracetamol); ASA, acetylsalicylic acid (aspirin); CASA, computer-assisted semen analyses; COX1–3, cyclooxygenase 1–3; Dnmt, *de novo* DNA methylase; dpc, days postcoitum; dpp, days postpartum; ECM, extracellular matrix; EdU, 5-ethynyl-2'-deoxyuridine; eSpd, elongated spermatid; F0AI, mice exposed *in utero* to *N*-acetyl-*p*-aminophenol + ibuprofen; F0WT, mice not exposed *in utero* to *N*-acetyl-*p*-aminophenol + ibuprofen; F1AI-AI, male F0AI × female F0AI mice; F1AI-WT, male F0AI × female F0WT mice; F1WT, male F0WT × female F0WT mice; F1WT-AI, male F0WT × female F0AI mice; FACS, fluorescence-activated cell sorting; 5-mC, 5-methylcytosine; GEO, Gene Expression Omnibus; IBU, ibuprofen; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NSAID, nonsteroidal antiinflammatory drug; PAS, periodic acid–Schiff; PG, prostaglandin; PGC, primordial germinal cell; qRT-PCR, quantitative RT-PCR; rSpd, round spermatid; SpgA/B, spermatogonia A/B; Spz, spermatozoa; STR, straightness; TXA2, thromboxane; UPLC, ultraperformance liquid chromatography–tandem mass spectrometry; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity

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In humans, genital malformations in newborn boys such as cryptorchidism and hypospadias as well as reproductive disorders in adults have been progressively increasing during the last 3 decades (1). These disorders may comprise a testicular dysgenesis syndrome with a common origin in fetal life and could be favored by exposure to environmental chemicals, such as pesticides, endocrine disruptors, or therapeutic drugs (2). Exposure *in utero* to endocrine disruptors can also have intergenerational adverse effects, particularly in gonadal germ cells (3), where they could lead to reduced semen quality and fertility (2). The critical window for exposure is the onset of fetal gonadal sex determination. This occurs during the epigenetic reprogramming of male gonocytes (4) that then generate the spermatogonia pool in the postnatal testis to support spermatogenesis throughout the adult reproductive life (5).

Nonsteroidal antiinflammatory drugs (NSAIDs) such as aspirin (ASA) and ibuprofen (IBU), and analgesic drugs such as *N*-acetyl-*p*-aminophenol (APAP; acetaminophen,

paracetamol) are widely used to treat inflammation and pain (6). APAP and IBU are among the most commonly prescribed drugs in the first trimester of pregnancy (7–9), and both APAP and NSAIDs can cross the placental barrier (10, 11). Epidemiologic studies reported that exposure to APAP or NSAIDs during the first and second trimesters of gestation is associated with an increased risk of cryptorchidism (6). Furthermore, in a large population-based mother–child cohort, an association between simultaneous maternal use of both APAP and NSAID and shorter anogenital distance was found, whereas APAP use alone did not affect anogenital distance in the male offspring (12). Experimental *in vivo/ex vivo* investigations in rodents (13–16) and *ex vivo* studies using human testicular explants (17–20) showed that nontherapeutic doses of these drugs cause mild or contradictory effects on testosterone secretion. Also, when administered to adult men, IBU disrupts the hypothalamus–pituitary–gonad axis, inducing an increase in LH secretion and a decrease in testosterone production; *ex vivo* studies using adult testicular explants confirmed the antiandrogenic action of IBU (21).

However, the consequences of APAP and NSAID exposure on the adult reproductive health are still largely unknown (22). A recent study showed the association between APAP and its metabolite *p*-aminophenol (quantified in urine) and reduced sperm quality in 500 male partners of couples planning for pregnancy (23). Moreover, in adult rats, long-term treatment with APAP at high doses impairs sexual competence, sperm count and quality, and fertility (24). Conversely, adult mouse sperm parameters are not affected after *in utero* exposure to low doses of ASA or IBU (25). Similarly, *in vivo* administration of APAP or indomethacin to pregnant rats during embryogenesis decreases the number of fetal germ cells and accelerates their embryonic differentiation but does not affect the fertility of adult male rats (26). In females, the number of primordial germ cells is reduced (27) and meiotic entry delayed (26) in fetal mouse and rat ovaries, respectively, leading to a decreased number of follicles in adult mouse ovaries (27) and reduced fertility in F1 and F2 rat females (26). In addition, exposure of *ex vivo* cultures of human embryonic ovaries to IBU induces a decrease in the germ-cell pool due to a decrease in their proliferation and an increase in their apoptosis (28).

NSAIDs and APAP target the enzymes cyclooxygenases 1–3 (COX1–3). COX1–3 catalyze the conversion of arachidonic acid into prostaglandin (PG) H₂, which is then converted into various PGs (PGE₂, PGD₂, PGI₂, PGF₂α) by the action of specific terminal PG synthases (29). Most PGs play multiple roles in embryonic and adult gonads and consequently in various aspects of male and female reproduction (30–32). Moreover, compounds that block the arachidonic acid cascade can inhibit testosterone synthesis and the development of male genitalia (33). Because prostanoid receptors and PG synthases (PGDS, PGES, and PGIS) are widely expressed in 11.5 d postcoitum (dpc) undifferentiated male gonads (34) and PGD₂ signaling is involved in the early steps of testis organogenesis [both somatic (35, 36) and germ-line cells (37)], embryonic gonads might be targets of these drugs. Therefore, using human-relevant doses (19, 38),

we investigated the effect of early *in utero* exposure (between 10.5 and 13.5 dpc), the period of gonad sex determination in mice that corresponds to the last part of the first trimester, 6 to 10 wk, of gestation in humans to APAP and NSAIDs as single drugs or for the first time in combination. Using the APAP + IBU combination, we found that germ-cell differentiation and maturation were affected in male embryonic and postnatal testes, leading to abnormal epididymal sperm parameters in adult animals directly exposed *in utero* (F0) and their offspring (F1 generation) when both parents were exposed. These data provide new findings on the negative effects of these pharmaceutical molecules, which are widely used by pregnant women, on gonad development and maturation and consequently on reproductive health.

MATERIALS AND METHODS

Animals and experimental protocols

For all experiments, wild-type 10.5 dpc CD1 females were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France). All animal experiments were conducted according to procedures approved by the Réseau des Animaleries de Montpellier (34-366 for B.B.-B.) and by the Regional Ethics Committee. Animals were kept and bred at the Institute of Human Genetics Animal Care Facility in controlled environmental conditions. APAP, ASA, and IBU stock solutions (500, 277, and 219 mM, respectively) (MilliporeSigma, Burlington, MA, USA) were prepared in ethanol and 10-fold diluted with PBS before oral administration between 10.5 and 13.5 dpc. For experiments with single drugs, 30 mg/kg APAP, 50 mg/kg ASA, and 15 mg/kg IBU (19, 20, 27, 39) or 9.7% ethanol (control) were administered daily at 2 times (150 μl by gavage, 6 h apart) (*n* = 6 experiments with 2 pregnant females for each condition). For experiments with 2 drugs [30 mg/kg/d APAP + 50 mg/kg/d ASA, and 30 mg/kg/d APAP + 15 mg/kg/d IBU or ethanol and APAP + APAP (60 mg/kg/d) as controls], the same doses for each drug were alternately administered every 3 h (4 times, 150 μl by gavage) (*n* = 4 experiments with 2 pregnant females for each condition). 5-Ethynyl-2'-deoxyuridine (EdU) (2.5 mM/150 μl) (Thermo Fisher Scientific, Waltham, MA, USA) was intraperitoneally injected in pregnant females 2 h before they were humanely killed and subsequent embryo dissection, and in 8 d postpartum (dpp) pups. Treated pregnant females gave birth to the F0 offspring (exposed *in utero*); then 8 wk old F0 males and 6 wk old F0 females (FOAI) were mated to generate F1 animals. Neither sibling nor cousin animals were crossed to avoid inbreeding artifacts.

Immunofluorescence

Mesogonads from embryos and from 8, 14, 21, and 30 dpp postnatal testes and 3 mo old adult testes from F0 and F1 animals (*n* = 6–10 animals/condition, each from independent exposed females or mating) were dissected. Fixed testes were embedded in paraffin, and sections were as previously described (35). Spreads were made from sperm suspension in Tris–citric–fructose buffer (*n* = 3 for each mating) and fixed with 4% paraformaldehyde (Sigma-Aldrich). Primary antibodies and dilutions used in immunofluorescence experiments [performed as previously described (34, 35)] included the following: rabbit-Sox9 [1:300, homemade (40)], rabbit-Amh [1:400, a gift from N. diClemente (University of Paris Diderot) (41)], rabbit L-Pgds [1:400 homemade (35)], rabbit Vasa/Mvh (1:300, AB4330; MilliporeSigma), mouse Vasa/Mvh (1:2000, ab27591; Abcam,

Cambridge, MA, USA), rabbit-Lin28a (1:400, 11724-1-AP; Proteintech, Rosemont, IL, USA), rabbit-5-methylcytosine (5-mC; 1:1500, 28692; Cell Signaling Technology, Danvers, MA, USA), CoupTFII/Nr2f2 (1:300, H7147; R&D Systems, Minneapolis, MN, USA), mouse- α -tubulin and -acetylated α -tubulin clone 6-11-B1 (1:1000, T9026, T6793; MilliporeSigma), and rabbit-histone H4 K8acetyl (1:1000, ab15823; Abcam). The appropriate secondary antibodies (1:1000, Alexa-Ig; Molecular Probes, Eugene, OR, USA) were used. EdU-positive cells were detected using the Click-iT EdU assay according to the manufacturer's instructions (Thermo Fisher Scientific). Images were captured with a Zeiss Axioimager ApoTome microscope (Carl Zeiss GmbH, Jena, Germany) and processed with Zen software (Zeiss).

RNA isolation and real-time PCR analysis

Embryonic gonads of 2 litters (2 pregnant mice/condition and experiment) were separated from the mesonephros and pooled by sex. Specifically, 13 to 24 gonads were processed for each experiment ($n = 4$). For postnatal stages, testes ($n = 6$ –10/condition) were pooled to get 3 independent groups; control and F0AI adult testes were processed independently ($n = 4$ /condition from different animals). RNA was extracted from gonads using Trizol (Thermo Fisher Scientific). Real-time reverse transcription PCR was performed as previously described (35). For each experiment, 2 independent reverse transcriptase (SuperScriptIV; Thermo Fisher Scientific) reactions and 3 PCR runs were performed on a LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA). *Rps29* was used as the reference gene for data normalization of embryonic gonads (42) and *18S* for data normalization of postnatal/adult samples. Primer sequences are as follows: *Sox9*, forward, 5'-TCGGACACGGAGAACACC-3', reverse, 5'-GCACACGGGGAACCTTATCTT-3'; *Sox2*, forward, 5'-TGGGCTCTGTGGTCAAGTC-3', reverse, 5'-TGATCATGTC-CCGGAGGT-3'; *Cdkn1a* (*p21*), forward, 5'-AACATCTCAG-GCCGAAA-3', reverse, 5'-TGGCTTGGAGTGATAGAAA-3'; *Cdkn1c* (*p57*), forward, 5'-CAGGACGAGAATCAAGAGCA-3', reverse, 5'-GCTTGGCGAAGAAGTCGT-3'; *L-Pgds*, forward, 5'-GGCTCCTGGACACTACACCT-3', reverse, 5'-GCACACG-GGGAACCTTATCTT-3'; *mPges-1*, forward, 5'-GCACATGCT-GGTCATCAAG-3', reverse, 5'-ACGTTTCAGCGCATCCTC-3'; *H-Pgds*, forward, 5'-CACGCTGGATGACTTCATGT-3', reverse, 5'-AATTCATTGAACATCCGCTCTT-3'; *mPges-2*, forward, 5'-CCCAGGAAGGAGACAGCTT-3', reverse, 5'-AGGTAGGTC-TTGAGGGCACTAAT-3'; *c-Pges*, forward, 5'-CGAATTTTGA-CCGTTTCTCTG-3', reverse, 5'-TGAATCATCATCTGCTCC-ATCT-3'; *Akr1b3*, forward, 5'-ACAATAAACTACAGCCCA-GGTG-3', reverse, 5'-TCGCTGCTCACCTCAAAGT-3'; *Akr1b7*, forward, 5'-CCACCTTCGTGGAAGTCAAG-3', reverse, 5'-CTTGGCCTGGGGAAGACT-3'; *17 β Hsd3*, forward, 5'-AATA-TGTCACGATCGGAGCTG-3', reverse, 5'-GAAGGGATCCG-GTTCAGAAT-3'; *15-Pgdl*, forward, 5'-GCAGGCGTGAACA-ATGAGA-3', reverse, 5'-GGTCCCCTGATAACAGAAACC-3'; *Rps29*, forward, 5'-TGAAGGCAAGATGGGTACAC-3', reverse, 5'-GCACATGTTAGCCCGTATT-3'; *18S*, forward, 5'-GATCCATTGGAGGGCAAGTCT-3', reverse, 5'-CCAAGAT-CCAAGTACGAGCTTT-3'; *3 β Hsd*, forward, 5'-GACCAGAA-ACCAAGGAGGAA-3', reverse, 5'-GCACTGGGCATCCAG-AAT-3'; *Cyp17a1*, forward, 5'-CATCCCCACACAAGGCT-AACA-3', reverse, 5'-CAGTGCCAGAGATTGATGA-3'; *Ar*, forward, 5'-CCAGTCCCAATTGTGTCAAA-3', reverse, 5'-TC-CCTGCTGTCCAAACG-3'; *StaR*, forward, 5'-CCACCTTCGT-GGAAGTCAAG-3', reverse, 5'-CTTGGCCTGGGGAAGACT-3'; *Scs*, forward, 5'-AAGTATGGCCCCATTTACAGG-3', reverse, 5'-TGGGGTCCACGATGTAACT-3'; *Lhr*, forward, 5'-GAT-GCACAGTGGCACCTTC-3', reverse, 5'-CCTGCAATTTG-GTGAAGAG-3'; *Amh*, forward, 5'-GGGGAGACTGGAGA-ACAGC-3', reverse, 5'-AGAGCTCGGGCTCCATA-3'; *Tnp1*, forward, 5'-AGCCGCAAGCTAAAGACTCA-3', reverse, 5'-

CGTAATTCGCACTTGCAT-3'; *Tnp2*, forward, 5'-GAGCC-TTCCCACCACTCAT-3', reverse, 5'-TGCCTGGTTACTGGT-GTGACT-3'; *Prm1*, forward, 5'-GACAGCCCCACAAAATT-CCAC-3', reverse, 5'-CAGAGCAGGGGACACCAC-3'; *Prm2*, forward, 5'-GAAGCGGAGGAGACACTC-3', reverse, 5'-CTCCTCTTCGGGATCTTCT-3'; *Dmmt3L*, forward, 5'-AA-CCGACGGAGCATTGAA-3', reverse, 5'-CCGAGTGTACAC-CTGGAGAGT-3'.

Fluorescence-activated cell sorting analysis

Control and F0AI postnatal testes ($n = 5$ –8) were incubated with collagenase (Roche Diagnostics) as previously described (37) and DNase (Thermo Fisher Scientific) at 37°C for 30 min. Dissociated testis cells were fixed with 1% paraformaldehyde at room temperature for 20 min and permeabilized in a PBS solution with 0.4% NP40, 1% bovine serum albumin, and 10 U RNaseA (Roche Diagnostics). Germ cells were stained with anti-rabbit Vasa/Mvh (1:300, AB4330; MilliporeSigma) or rabbit-Lin28a (1:400, 11724-1-AP; Proteintech) antibodies and Sertoli cells with rabbit anti-Sox9 antibody, followed by a goat anti-rabbit Alexa 555 secondary antibody (1:1000; Thermo Fisher Scientific). Samples were resuspended in 500 μ l of PBS containing DAPI (1 mg/ml; MilliporeSigma) to label the DNA. Mvh-, Lin28a-, and Sox9-positive and -negative cell populations were gated, and DAPI staining was evaluated in each population. A minimum of 10,000 cells was analyzed for each sample using a MACS Quant Analyzer (Miltenyi Biotec, San Diego, CA, USA). Data were analyzed by FlowJo software (Treestar, Ashland, OR, USA).

NSAID, PG, and steroid measurements by liquid chromatography–tandem mass spectrometry

Pregnant females were humanely killed and embryonic gonads (without mesonephros) and liver dissected 30 min after the second gavage of the day, APAP, ASA, and IBU, respectively, for APAP + APAP, APAP + ASA, and APAP + IBU treatments. For each condition, APAP, ASA, and IBU were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in plasma from pregnant mice ($n = 2$) and in livers from male 13.5 dpc embryos (100–310 mg from 2 littermates). Embryo livers were homogenized in water using the Precellys (Bertin Technologies, Montigny-le Bretonneux, France). Then 50 μ l of each plasma or liver sample was added to 200 μ l of acetonitrile with 2% of ammonia, vortexed for 15 s, and centrifuged at 4°C for 15 min. Supernatants were transferred to fresh tubes and evaporated to dryness using a Turbovap LV Evaporator (Biotage, Hengoed, United Kingdom). Extracts were resuspended in 50 μ l of water with 0.2% ammonia. IBU and APAP were quantified by linear regression with $1/x^2$ weighing and a range from 25 to 5000 and 5 to 500 ng/ml, respectively. Salicylic acid was measured using quadratic regression with $1/x$ weighing and a range of 25 to 2500 ng/ml. Each sample (10 μ l) was injected in the LC-MS/MS system, which included a Waters Acquity ultraperformance liquid chromatography–tandem mass spectrometry (UPLC) system with an Acquity UPLC BEH phenyl 1.7 μ m, 2.1 $\times$ 100-mm column and a reversed-phase gradient over a run time of 5 min (Waters, Milford, MA, USA).

PGD₂, 15-desoxy 12, 14-PGJ₂, PGE₂, PGF₂ α , 6-keto-PGF₁ α (PGI₂), and thromboxane (TXA₂) were quantified in 13.5 dpc control and APAP + IBU-treated testes by LC-MS/MS. For this experiment, 200 control and 210 exposed testes were isolated from embryos of 15 controls and 15 APAP + IBU-treated pregnant females and then pooled. Testes were homogenized in methanol containing PGD₂-d4 and PGE₂-d4 as internal standards using the Precellys device (Bertin Technologies). Homogenates were centrifuged at 20,000 g for 10 min, and supernatants

were evaporated and resuspended in 100 μ l of 0.1% acetic acid in water/acetonitrile/isopropanol, 80/18/2, v/v/v. PGD₂, 15-desoxy 12,14-PGJ₂, PGE₂, PGF_{2a}, PGI₂, 6-keto-PGF1 α , and TXA₂ were quantified by linear regression with 1/ x^2 weighing and a range from 1 to 500 ng/ml. Ten microliters of extract was injected in the LC-MS/MS system, which included a Waters Acquity UPLC system with an Acquity UPLC BEH Shield 1.7 μ m, 2.1 $\times$ 100-mm column, and a reversed-phase gradient with a run time of 5 min. The initial conditions were as follows: mobile phase A (0.1% acetic acid) and mobile phase B (acetonitrile/isopropanol, 90/10) with a column temperature of 50°C and a flow rate of 0.400 ml/min between 0 and 2 min and 0.5 ml/min between 2 and 5 min.

Steroids (testosterone and its precursors 17 α -hydroxy progesterone and androstenedione) were quantified in 3 mo old control ($n = 5$), F0AI ($n = 5$) and F1AI-AI ($n = 6$) testes by LC-MS/MS. Adult testes were homogenized in methanol containing mixed internal standards (²H₇-delta4-androstenedione, ²H₄-testosterone, and ²H₈-17-hydroxyprogesterone). After evaporation, dried residues were resuspended in 600 μ l of methanol, loaded onto an Oasis Prime HLB (Waters) cartridge, and sequentially washed with 0.1% formic acid in 35% methanol and 0.1% ammonia solution in 35% methanol. Samples were eluted with 45 μ l of methanol and then diluted with 55 μ l of distilled water. After mixing, 30 μ l were injected for UPLC analysis.

Sperm analysis

The IVOS I computer-assisted semen analyses (CASA) system (Hamilton Thorne, Beverly, MA, USA) was used for semen concentration and motility assessment. Both cauda epididymis were immersed and chopped with small scissors into 100 μ l of Tris-citric-fructose buffer. After incubation at 37°C for 10 min for sperm release, sperm cells were collected from the epididymis tissue. A 4 μ l aliquot was placed into a standardized 4-chamber Leja counting slide (Leja Products, Nieuw-Vennep, The Netherlands). The IVOS settings chosen were those defined for mice sperm-cell analysis (by Hamilton Thorne): a total of 30 frames were captured at 60 Hz, with software settings as follows: cell detection with a minimum contrast of 50, a minimum cell size of 4 pixels, and a cell intensity of 80. The recorded CASA parameters include the average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement, motility (percentage), and sperm concentration (10⁶/ml). The cutoff value for progressive cells was as follows: for VAP (50 μ m/s) and for straightness (STR; 80.0%); slow cells were recorded as static and were assigned a VAP cutoff of 7.4 μ m/s and a VSL cutoff of 6.6 μ m/s. With an automated stage, 10 microscope fields were analyzed using the predetermined starting position within each chamber. Statistical analyses were performed using the mean of the 10 analyzed fields with at least 500 cells. The normality of the parameters was verified *via* the Shapiro-Wilk test, and a variance comparison analysis (ANOVA) was performed with the GLM procedure in SAS software (SAS Institute, Cary, NC, USA).

mRNA expression profiling and analysis

Cytoplasmic and mitochondrial rRNAs were removed from 275 ng of total RNA using the Ribo-Zero Gold Depletion Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The rRNA-depleted RNA was purified using the Agencourt RNAClean XP Kit (Beckman Coulter, Brea, CA, USA), and libraries were prepared with the TruSeq Stranded Total RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. This includes a first-strand synthesis, a second-strand synthesis, end repair, A tailing, adapter ligation, and a final PCR

step. Amplified libraries were purified using the Agencourt XP Kit (Beckman Coulter).

The RNA-Seq library quality was evaluated by on-chip electrophoresis (DNA 1000 chip) of 1 μ l of each sample on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library DNA concentration was estimated using Qubit 2.0 with the DNA HS Assay (Thermo Fisher Scientific). RNA-Seq libraries were pooled in 4-nM stocks, denatured, and diluted to 1.2 pM. Libraries were sequenced (75 bp reads) in a paired-end format on a NextSeq 500 instrument (Illumina).

The BCL files were converted to FastQ format by Bcl2fastq v.2.17 software (Illumina) for analysis. Sequence data were checked with the FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

Genome sequences and gene annotations were downloaded from Ensembl (GRCm38, release 84; ftp://ftp.ensembl.org/pub/release-84/fasta/mus_musculus/dna/, ftp://ftp.ensembl.org/pub/release-84/gff3/mus_musculus/). SAMtools v.1.3 (43, 44), Bowtie v.2.2.6 (45), TopHat v2.1 (46), and Cufflinks v.2.2.1 (47, 48) were downloaded (<http://www.htslib.org/>, <http://bowtie-bio.sourceforge.net>, <https://ccb.jhu.edu/software/tophat>, and <http://cole-trapnell-lab.github.io/cufflinks>, respectively) and installed on a Linux server following their software instruction manuals.

Paired-end reads were aligned to the reference genome (GRCm38) with Bowtie2 and TopHat. The Cufflinks suite of tools was used to quantify gene expression and for comparison of transcript expression levels. Results were visualized and analyzed with the R (R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org/>) package CummeRbund (<http://compbio.mit.edu/cummeRbund/>).

Statistical analysis

Statistical analyses were performed by Prism 7 (GraphPad Software, La Jolla, CA, USA). The Student's *t* test was used to compare 2 groups in independent experiments and the 1-way ANOVA test with Geisser-Greenhouse correction and the Tukey's *post hoc* test for multiple comparisons [real-time quantitative RT-PCR (qRT-PCR) experiments and cell counting]. Results were considered statistically significant at $P < 0.05$. For CASA data on sperm parameters, statistical analysis was performed by SAS 9.4 software (SAS Institute, Cary, NC, USA). Differences between mice groups were assessed by using the general linear model procedure (proc GLM) and considered significant at $P \leq 0.05$.

RESULTS

***In utero* exposure to NSAIDs and APAP impairs male germ-cell, but not somatic-cell, proliferation in mouse embryonic gonads**

In the mouse, Sertoli-cell differentiation starts shortly after the onset of *Sox9* expression, which is induced by the testis-determining factor SRY (49). Meanwhile, primordial germ cells that have reached the gonadal ridge by 10.5 dpc proliferate up to 13.5 dpc. Concomitant with pluripotency gene repression and mitotic arrest, primordial germ cells differentiate into prospermatogonia between 13.5 and 15.5 dpc (50). To evaluate the effect of NSAIDs or APAP exposure on these early events, 30 mg/kg/d APAP, 50 mg/kg/d ASA, 15 mg/kg/d IBU, or diluent alone (9.7% ethanol, control) was individually administered by gavage to pregnant mice between 10.5 and 13.5 dpc. This

period corresponds to the last part of the first trimester of gestation in humans (6–10 wk). Immunofluorescence analysis of 13.5 dpc male gonad sections using antibodies against Sox9 (Sertoli-cell marker) and Vasa/Mvh

(germ-cell marker) did not highlight any difference in testis cord formation after *in utero* exposure to each of the 3 drugs compared to control (**Fig. 1A**). Coimmunofluorescence analysis after EdU labeling showed that

Figure 1. Exposure to APAP, ASA, or IBU decreases germ-cell proliferation. **A**) Representative immunofluorescence images of testes from mouse 13.5 dpc embryos, mothers of which were treated with ethanol (control), APAP, ASA, or IBU between 10.5 and 13.5 dpc and pulsed with EdU at 13.5 dpc; Sox9 (Sertoli-cell marker) in red, Vasa/Mvh (germ-cell marker) in purple, and EdU in green (scale bar, 100 μ m). Arrows and arrowheads highlight proliferating EdU⁺ germ cells and EdU⁺ Sertoli cells, respectively. **B**) Quantification of Sox9⁺EdU⁺ and Mvh⁺ EdU⁺ cells in control and treated testes; data are percentage of proliferating EdU⁺ cells among all Sox9⁺ or all Mvh⁺ cells. **C**) Quantification (percentage) of Ki-67⁺ cells among all Mvh⁺ cells in 13.5 and 14.5 dpc control and 13.5 dpc *in utero*-exposed testes. **B**, **C**) Values are means $\pm$ SEM for $n = 6$ gonads/group; 700 to 2100 counted cells for each data point. **D**) Percentage of proliferating EdU⁺ cells among all Mvh⁺ cells in control and *in utero*-exposed testes at 12.5, 13.5, or 14.5 dpc; pregnant females were pulsed with EdU 2 h before dissection. Values are means $\pm$ SEM for $n = 3$ –6 gonads/group and 80 to 870 counted cells for each point. **E**) qRT-PCR analysis of *Oct4*, *Nanog*, *Sox2*, and *Sox9* expression levels normalized to *Rps29* expression and presented as percentage of expression in control 13.5 dpc testes (set to 100). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (**B**–**E**).

the percentage of S-phase cells was significantly reduced in germ cells (Mvh⁺EdU⁺), but not in Sertoli cells (Sox9⁺EdU⁺), in *in utero*-exposed gonads compared to control (Fig. 1A, B). Accordingly, the proliferative index of germ cells (Ki-67⁺Mvh⁺) was significantly lower in *in utero*-exposed than in control 13.5 dpc gonads (Fig. 1C). However, the tubular diameter, the total number of Sertoli or germ cells per tubule, and the apoptotic index did not vary between *in utero*-exposed and control 13.5 dpc gonads (unpublished observations). Moreover, the percentage of S-phase germ cells (Mvh⁺EdU⁺) in *in utero*-exposed 12.5 dpc gonads was comparable to that of 13.5 dpc controls (Fig. 1D). This suggests that *in utero* exposure to APAP, ASA, or IBU might advance germ-cell mitotic arrest. In agreement, expression of the pluripotent genes *Sox2* and *Nanog* was significantly lower in APAP (but not in ASA or IBU) exposed gonads compared to controls (Fig. 1E). However, expression of the Sertoli differentiating marker *Sox9* is increased only in 13.5 dpc gonads exposed *in utero* to ASA (but not to APAP or IBU) (Fig. 1E).

Because NSAIDs (particularly ASA and IBU) and APAP are used together by nearly 4% of pregnant women (12), the APAP + ASA and APAP + IBU combinations were administered to pregnant mice between 10.5 and 13.5 dpc to evaluate their effects on the differentiation of testis somatic and germ cells in male embryos. Controls received ethanol or a double dose of APAP (APAP + APAP). Immunofluorescence analysis using antibodies against Sox9 and Vasa/Mvh indicated that testis cords were normally formed in testes from *in utero*-exposed and control 13.5 dpc male embryos (Fig. 2A). However, coimmunofluorescence experiments after *in vivo* EdU labeling of pregnant females showed that the percentage of S-phase germ cells (Mvh⁺EdU⁺), but not of Sertoli cells (Sox9⁺EdU⁺), was significantly reduced in gonads from 13.5 dpc embryos exposed to APAP + ASA or APAP + IBU compared to controls (Fig. 2A, B). Exposure to APAP alone (APAP + APAP) did not have any significant effect in both cell types, possibly because of the accelerated APAP metabolism in liver in the presence of high ethanol concentration (diluent), resulting in a weaker concentration of available drug, as previously described (51). Moreover, the germ-cell proliferation index (Ki-67⁺Mvh⁺ cells) was significantly higher in 13.5 dpc gonads from embryos exposed *in utero* to APAP + ASA or APAP + IBU than in controls, suggesting an accumulation of germ cells in the G₁ phase of the cell cycle (Fig. 2C). Finally, *Sox9* was significantly up-regulated and *Sox2* (pluripotency gene) down-regulated in APAP + IBU testes. *Cdkn1a* and *Cdkn1c*, 2 genes that encode the cell cycle inhibitors p21 and p57, were up-regulated in APAP + ASA and APAP + IBU testes, respectively (Fig. 2D). Similar to the results with single drugs, these data are consistent with early embryonic germ-cell mitotic arrest and concomitant decreased cell proliferation in APAP + ASA and APAP + IBU embryonic testes. They also suggest that APAP and IBU may interact with each other to affect cell differentiation in the embryonic testis.

To confirm that APAP and NSAIDs can cross the placental barrier (10, 11), first the plasma concentrations of

APAP, ASA, and IBU were measured in pregnant mice humanely killed 30 min after APAP, ASA, and IBU treatment (1070, 28,600, and 520 ng/ml, respectively) (Fig. 2E). APAP plasma concentration was similar to what reported in a human testis xenograft mouse model after administration of therapeutic doses of APAP (740 ng/ml) (19). The concentration of APAP, ASA, and IBU in male embryo livers (611, 4648, and 105 ng/g, respectively) confirmed that embryos were exposed to these chemicals after oral administration to their mothers (Fig. 2E). Residual APAP was detected in the liver of control embryos from mothers treated with ethanol (61.4 ng/g) or PBS (35 ng/g) (Fig. 2E) due to residual APAP in the drinking water (51 ng/L) of our animal care facility (unpublished observations), which is consistent with data reporting APAP presence in the urine of various populations (52). Because NSAIDs and APAP can pass through the placental barrier after oral administration (Fig. 2E) and target COXs, PG production was assessed in control and APAP + IBU *in utero*-exposed testes. As a result of its short half-life, PGI₂ production is typically monitored by measuring the 6-keto-PGF1 α metabolite, which is a biologically inactive hydration product (53). PGD₂, PGE₂, and 6-keto-PGF1 α production was decreased by 22, 37, and 63%, respectively, in 13.5 dpc APAP + IBU exposed testes compared to control (Fig. 2F). However, 15-desoxy-PGJ₂, an active PGD₂ metabolite, PGF2 α , and TXA₂ could not be detected in control and exposed testes. qRT-PCR analysis of genes involved in PG synthesis showed weak expression of prostaglandin-endoperoxide synthase 2 (*Cox-2*), prostaglandin E synthase (*Pges*)-1, and aldo-keto reductase family 1, member B7 (*Akr1b7*) (encoding one of the PGF2 α synthases) and stronger expression of the genes encoding the other PG synthases and of 15-hydroxyprostaglandin dehydrogenase (*15-Pgdh*), encoding the key PG catabolic enzyme in 13.5 dpc control testes (Fig. 2G). Upon *in utero* APAP + IBU exposure, hematopoietic prostaglandin D synthase (*H-Pgds*), prostaglandin E synthase (*Pges*)-1, -2, and aldo-keto reductase family 1, member B7 (*Akr1b3*) (a second PGF2 α synthase) were significantly up-regulated, and *15-Pgdh* down-regulated, compared to control testes (Fig. 2G). This could explain the relatively low decrease in PGD₂ and PGE₂ production (54). Conversely, APAP + IBU exposure had no effect on prostaglandin-endoperoxide synthase 1 (*Cox-1*), *Cox-2*, prostaglandin D2 synthase (*L-Pgds*), prostaglandin E synthase 2 (*c-Pges*), and *Akr1b7* expression (Fig. 2G).

***In utero* exposure to APAP and IBU induces early male germ-line differentiation**

Transcriptome analysis was then performed to identify the genes deregulated in 13.5 dpc male gonads after *in utero* exposure to APAP and IBU or ASA. Compared to controls, 40, 20, and 122 genes were differentially expressed in APAP + APAP (3 up- and 37 down-regulated), APAP + ASA (5 up- and 15 down-regulated) and APAP + IBU (103 up- and 19 down-regulated) testes ($P = 5 \times 10^{-5}$), respectively (Fig. 3A and Supplemental Table S1). However, only 5 differentially expressed genes were common to the 3 data sets (Fig. 3B). Categorization of the differentially

Figure 2. *In utero* exposure to APAP and NSAIDs decreases germ-cell proliferation. **A**) Representative immunofluorescence microscopy images of 13.5 dpc testes from embryos exposed *in utero* to ethanol (control), APAP + APAP, APAP + ASA, or APAP + IBU between 10.5 and 13.5 dpc and pulsed with EdU at 13.5 dpc; Sox9 (Sertoli-cell marker) in red, Vasa/Mvh (germ-cell marker) in purple, and EdU in green (scale bar, 100 μ m). Insets show EdU⁺ germ cells (arrows) and Sertoli cells (arrowheads). **B**) Quantification of Sox9⁺EdU⁺ and Mvh⁺EdU⁺ cells in control and treated testes; data are represented as percentage relative to all Sox9⁺ and Mvh⁺ cells. **C**) Percentage of Ki-67⁺ cells among all Mvh⁺ cells in 13.5 and 14.5 dpc control and 13.5 dpc exposed testes. **B, C**) Values are means $\pm$ SEM for $n = 15$ –22 gonads in each group and 500 to 2000 counted cells for each data point. **D**) qRT-PCR analysis of *Sox9*, *Cdkn1a*, *Cdkn1c*, and *Sox2* expression in 13.5 dpc testes. Data were normalized to *Rps29* expression and are presented as percentage of expression in control testes (set to 100). Data are means $\pm$ SEM, $n = 4$ experiments. **E**) APAP, ASA, and IBU concentrations in plasma of control, PBS, APAP + APAP, APAP + ASA, and APAP + IBU treated pregnant females and in liver of their male embryos (mean $\pm$ SEM of 2 experiments). **F**) Dosage of PGD₂, PGE₂ and 6-keto-PGF1 α (PGI₂) by mass spectrometry in 13.5 dpc control and APAP + IBU exposed testes ($n = 1$ pool of 200 and 210 gonads, respectively). **G**) qRT-PCR analysis of genes encoding factors involved in PG synthesis in 13.5 dpc control and APAP + IBU exposed testes; data were normalized to *Rps29* expression and are shown as means $\pm$ SEM; $n = 3$ experiments. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (**B–D, G**).

Figure 3. Exposure to APAP and IBU leads to early germ-cell differentiation through early 5-mC deposition and increased ECM protein secretion. **A)** Number of genes that are expressed in embryonic testes and significantly deregulated (at least 1.5-fold change) in APAP + APAP, APAP + ASA, or APAP + IBU exposed 13.5 dpc testes compared to control. Number of down- or up-regulated genes is indicated in red. These data are accessible through GEO Series accession number GSE89061 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89061>). **B)** Venn diagram showing overlap of genes that are differentially expressed in 3 different experimental conditions. **C)** Coimmunofluorescence analysis of DNA methylation (anti-5-mC antibody, red) in germ cells (anti-Vasa/Mvh antibody, green) in 13.5 and 14.5 dpc controls and 13.5 dpc APAP + IBU exposed testes; nuclei are stained with Hoechst (Hst, blue). Arrows highlight 5-mC staining in germ cells; insets show high-magnification images of boxed areas. **D)** Quantification of 5-mC⁺ germ cells (percentage of all Mvh⁺ cells) in 13.5 dpc control and APAP + IBU exposed testes and 14.5 dpc control testes. Values are means $\pm$ SEM for $n = 10$ gonads in each group and 50 to 120 counted germ cells/gonad; ns, not significant. **** $P < 0.0001$. **E)** PAS staining of 13.5 dpc control and APAP + IBU exposed testes. Arrows highlight glycoproteins deposits within testis cords. Scale bars, 25 μ m (**C**) and 50 μ m (**D**).

expressed genes using the ToppGene program (55) and Gene Ontology analysis showed that APAP + APAP and APAP + ASA influenced similar molecular, biologic, and cellular functions/pathways (Supplemental Table S2).

Conversely, the APAP + IBU combination affected genes involved in transcriptional regulation activity, extracellular matrix (ECM) regulation, organ morphogenesis, and the TGF- β signaling pathway (Supplemental Table S2), suggesting different mechanisms of action for the APAP + IBU combination compared to APAP or APAP + ASA.

We focused on the analysis of the 122 transcripts (96 genes) deregulated by *in utero* exposure to APAP + IBU using data from the National Center for Biotechnology Information Gene Expression Omnibus (GEO; Bethesda, MD, USA; <https://www.ncbi.nlm.nih.gov/geo/>) data set browser (data set record GDS3995) (56). In this group, 14 female-specific and 13 male-specific genes were up-regulated (Supplemental Table S3), as were several homeobox genes (Supplemental Tables S2 and S3). Moreover, *Sox2* (0.53-fold), the nodal male inhibitor *Lefty1* (*left right determination factor 1*) (0.48-fold) that is required for pluripotency maintenance (57) and *Sohlh2* (*spermatogenesis and oogenesis specific basic helix-loop-helix 2*) (0.56-fold) that encodes a c-Kit regulator in the germ line (58) were among the 12 down-regulated genes (Supplemental Table S3). Conversely, 2 main testis-differentiating genes [*Sox9*, >1.6-fold; and *Dnmt3L* (*DNA (cytosine-5-)-methyltransferase 3-like*), >3.5-fold, which encodes the enzyme involved in male germ-line reprogramming] were up-regulated (Supplemental Tables S1 and S3). Induction of expression of *de novo* DNA methylases (Dnmts), including *Dnmt3L*, in male germ cells coincides with the reestablishment of DNA methylation (5-mC) at 14.5 dpc. Particularly, CpG island methylation of the *Sox2* and *Nanog* promoters (59) occurs concomitantly with germ-line differentiation onset (60). Immunofluorescence analysis with an anti-5-mC antibody showed that in 13.5 dpc APAP + IBU gonads, 6% of germ cells (Mvh⁺) were 5-mC positive, as in 14.5 dpc control gonads. Conversely, methylation could not be detected in 13.5 dpc control testes (Fig. 3C, D). This suggests that APAP + IBU exposure promotes earlier germ-line 5-mC deposition through *Dnmt3L* expression up-regulation. *In utero* APAP + IBU exposure also deregulated 22 genes that encode ECM proteins (Supplemental Table S4), particularly different collagen types (*Col2a1*, *Col9a1*, *Col11a1-a2*, *Col12a1*), and glycoproteins or proteoglycans such as aggrecan (*Acan*), versican (*Vcan*), lumican (*Lum*), matrilin3 (*Matn3*), testatin (*Cystatin9*, *Cst9*), and keratocan (*Kera*), most of which are directly activated by Sox9 in chondrocytes (61) and embryonic testis (62). In agreement, periodic acid-Schiff (PAS) staining showed that glycoprotein accumulation within the testis cords was significantly higher in APAP + IBU than in control 13.5 dpc gonads (Fig. 3E).

***In utero* exposure to APAP and IBU leads to impaired production of spermatozoa in F0 and reduced sperm quality in F1 adults**

To evaluate whether the observed embryonic phenotype affects fertility in adult males, 8 wk old male and 6 wk old female mice that were exposed *in utero* to APAP + IBU (F0AI) or not (F0WT) were mated to produce F1 animals (Fig. 4A), as follows: male F0AI $\times$ female F0WT (F1AI-WT) ($n = 7$); male F0WT $\times$ female F0AI (F1WT-AI) ($n = 15$);

Figure 4. Sperm parameters of F0 mice and F1 adult mice are impaired after *in utero* exposure to APAP and IBU. **A**) Schematic representation of F0 mating experiments. **B, C**) Number of Spz per both caudal epididymis (**B**) and percentage of motility (**C**) in 3 mo old control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI animals ($n = 8-10$ animals/group). Horizontal middle line represents mean sperm number and motility percentage; ns, no significant difference with control animals. **** $P < 0.0001$. **D**) Content of testosterone and its precursors 17 α -hydroxy progesterone and androstenedione were measured in control, F0AI, and F1AI-AI (continued on next page)

male F0AI $\times$ female F0AI (F1AI-AI) ($n = 19$); male F0WT $\times$ female F0WT (F1WT) ($n = 6$). All females were mated within a week; the frequency of full-term pregnancies (100%) and the average litter size (between 11 and 18 pups) were comparable, indicating that APAP + IBU exposure did not affect the reproductive ability of adult F0 animals. Because the phenotypes of F1WT and F0WT animals and testes were similar, data from these 2 groups are referred as control in the next experiments.

Quantification using the CASA system of cauda epididymal sperm of 3 mo old F0 and F1 adult males showed that F0AI mice, but not F1 animals, had a significant lower sperm count (19% decrease) than control mice [mean: 236×10^6 vs. 294×10^6 spermatozoa (Spz)/ml, $P = 0.0016$, control: $n = 10$, F0AI: $n = 9$, which corresponds to a production of 23 to 29×10^6 Spz/animal] (Fig. 4B). The number of Spz in F1AI-WT mice (mean: 228×10^6 Spz/ml) was also reduced, but the difference with control mice was not significant ($P = 0.11$). Analysis of the sperm motion parameters with the CASA system in F0 and F1 adult males showed significantly lower mean values for most motility parameters in F1AI-AI sperm compared to controls, including global motility, which was decreased by 40% ($P < 0.05$, $n = 8-10$) (Fig. 4C and Table 1), and VAP, VSL, and VCL (Table 1). Conversely, lateral head displacement and STR were not affected (Table 1). However, sperm motility was not affected in F0AI, F1WT-AI, and F1AI-WT mice. *In utero* exposure suppresses intratesticular testosterone by 80% and its precursors, 17α -hydroxy progesterone (80%) and androstenedione (60%), in F0AI adult animals compared to controls (Fig. 4D). Normal steroid production was recovered in 8 wk old F1AI-AI animals (Fig. 4D). These data suggest complex effects of *in utero* exposure to APAP + IBU, first acting on spermatogenesis in exposed F0AI animals, possibly through a deficit in testosterone production, and second affecting sperm quality in their offspring when both parents have been exposed *in utero* (F1AI-AI animals). This defect led to subfertility in 6 mo old F1AI-AI males that mated with a control female after more than 1 wk (66%, $n = 4$ of 6) (Fig. 4E); however, the litter size was similar to that of control males (Fig. 4E).

PAS staining indicated that overall, the histology of seminiferous epithelium in 3 mo old F0 and F1 testes was normal, with the different stages of the seminiferous epithelium cycle represented (Fig. 4F). However, in F1AI-AI seminiferous tubules, abnormal germ-cell characteristics were observed, such as atypical presence of multinucleated

cells, sloughed or clustered cells into the lumen, and vacuolization of the basal compartment (Fig. 4G). Furthermore, in F0AI and F1 adult testes, the interstitial compartment was larger than in controls (Fig. 4F). Accordingly, immunofluorescence analysis using antibodies against CoupTFII, a Leydig stem-cell marker (63), and L-Pgds, an adult Leydig cell (ALC) marker (31, 64), indicated that the number of Leydig stem cells (Fig. 4H, I) and ALCs (Fig. 4H) was significantly higher in F0 and F1 adult testes compared to controls. Quantification of Leydig-cell-specific mRNA transcripts showed a significant increased expression of the genes encoding steroidogenic acute regulatory protein (*Star*), cholesterol side-chain cleavage enzyme (*Scs*, *Cyp11a1*), 3β -hydroxysteroid dehydrogenase (3β -Hsd), and 17α -hydroxylase/ $17,20$ lyase (*Cyp17a1*) in F0AI testes compared to controls. The expression of the genes encoding luteinizing-choriogonadotropic hormone receptor (*LhR*) and 17β -hydroxysteroid dehydrogenase3 (*Hsd17b3*) was also increased in F0AI testes, but the difference with control mice was not significant (Fig. 4I). All together, these data show a significant alteration of testis morphology/function and suggest an hyperplasia of the Leydig cell lineage in *in utero*-exposed F0AI and also in F1 animals.

Sperm count reduction in *in utero*-exposed mice could be caused by reduction of spermatogonia A population

During early postnatal life, embryonic gonocytes/prospermatogonia differentiate into spermatogonia. This differentiation process is essential for the establishment of the spermatogonia A (SpgA) pool comprising the spermatogonial stem cells and consequently for fertility. The decrease in sperm count in F0 animals suggested that *in utero* exposure to APAP + IBU could interfere with the postnatal development of germ cells and adult spermatogenesis. To test this hypothesis, testes from F0 animals were analyzed during the first wave of spermatogenesis, at meiosis onset (8 dpp), when the germ-line population includes SpgA, differentiating spermatogonia B (SpgB), and some preleptotene spermatocytes, at 14 dpp at the time of meiotic prophase I (spermatocytes), at 21 dpp when postmeiotic cell (round spermatid, rSpd) start to appear, and at 30 dpp when spermatids begin to elongate (elongated spermatid, eSpd) (5).

testes ($n = 5-6$ testes/group). Levels of steroids are expressed in ng/mg testis; values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. E) Fertility of 6 mo old F1AI-AI males. Number of weeks (1-2 or 3) for mating and litter size after full-term pregnancies are shown for control ($n = 5$) and F1AI-AI ($n = 6$) males. Values are means $\pm$ SEM. * $P < 0.05$. F) PAS-stained transversal sections of seminiferous tubules from 3 mo old testes. White arrows indicate interstitial compartment. G) Enlarged panels of F1AI-AI sections. Yellow arrow shows clustered cells into lumen, yellow arrowheads multinucleated cells and sloughed cells in lumen, and asterisk shows vacuolization of basal compartment. H, I) Leydig-cell population in 3 mo old testes was identified (H) and quantified (I) by immunofluorescence with anti-L-Pgds (in red) and anti-CoupTFII (green) antibodies (arrows), and nuclei were stained with Hoechst (HST, blue); insets show representative images of interstitial compartment with L-Pgds (red) and CoupTFII (green) positive cells. TC, testis cord. Number of Leydig stem cells (CoupTFII⁺) in interstitial space (between 3 tubules) is represented as means $\pm$ SEM for $n = 5$ testes/group and $n = 5$ representative pictures/testis (I). J) qRT-PCR analysis of *Sox9*, *LhR*, *StaR*, *Scs* (*Cyp11a1*), 3β -Hsd, 17α -hydroxylase (*Cyp17a1*), and 17β -Hsd3 expression in 3 mo old F0AI testes ($n = 3$). Data were normalized to *18S* expression and are presented relative to expression in control testes (set to 1). Data are means $\pm$ SEM ($n = 4$ experiments). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$. Scale bars, 200 μ m (F), 100 μ m (G, H).

TABLE 1. *Sperm motility analysis*

Characteristic	Control (mean $\pm$ sd)	F0AI (mean $\pm$ sd)	F1WT-AI (mean $\pm$ sd)	F1AI-WT (mean $\pm$ sd)	F1AI-AI (mean $\pm$ sd)
Motility (%)	52.75 $\pm$ 15.8	50.33 $\pm$ 13.2	48.55 $\pm$ 11.1	53.43 $\pm$ 10.5	34.25 $\pm$ 9.7***
VAP (μ m/s)	79.02 $\pm$ 13.2	88.84 $\pm$ 13.3	73.28 $\pm$ 7.3	80.22 $\pm$ 9.2	66.83 $\pm$ 8.6***
VCL (μ m/s)	156.46 $\pm$ 24.3	177.55 $\pm$ 23	146.96 $\pm$ 15	161.48 $\pm$ 16.1	134.54 $\pm$ 16***
VSL (μ m/s)	48.61 $\pm$ 7.71	54.39 $\pm$ 8.3	45.53 $\pm$ 5	49.47 $\pm$ 6.3	41.38 $\pm$ 5.8***
STR (%)	58.5 $\pm$ 1.39	58.05 $\pm$ 0.52	59.38 $\pm$ 1.4	58.68 $\pm$ 1.4	59.2 $\pm$ 1.9
Lateral head displacement (μ m)	8.82 $\pm$ 0.34	9.03 $\pm$ 0.37	8.5 $\pm$ 0.39	8.63 $\pm$ 0.49	8.43 $\pm$ 0.9

*** $P < 0.005$ ($n = 8-10$ testes/group).

Body weight and anogenital distance index (65) were not significantly different in F0AI animals and controls at different time points after birth (Supplemental Fig. S1A, B), which suggests a normal level of fetal androgen exposure in F0 embryos. Similarly, staining with DAPI (a DNA intercalating agent) and fluorescence-activated cell sorting (FACS) analysis revealed that the cell-cycle distribution and 1N, 2N, and 4N DNA content of testis cells was not affected by *in utero* exposure (Supplemental Fig. S1C). However, incubation with anti-Vasa/Mvh (germ-cell marker) or Lin28a [SpgA marker (66)] antibodies and sorting by FACS showed that the number of germ cells and SpgAs was significantly decreased in F0AI testes at all postnatal stages (except at 30 dpp for Vasa/Mvh⁺ cells) (Fig. 5A, B). Immunofluorescence with a specific anti-Lin28a antibody showed a significant decrease in the number of the Lin28a⁺ cells per tubule in F0AI testes compared to controls (Fig. 5C and Supplemental Fig. S2A), confirming that SpgA number is affected in F0AI animals. In F1 testes, the SpgA number was significantly lower only in 8 and 14 dpp F1AI-WT testes (Fig. 5D). This suggests a transient intergenerational effect of *in utero* exposure to APAP-IBU on SpgA number.

To analyze meiosis progression, EdU was injected in 8 dpp control and F0AI pups, and animals were humanely killed at 8 dpp (2 h after injection) and at 14, 21, and 30 dpp (1, 2, and 3 wk after EdU injection, respectively). At all stages except 8 dpp, the number of EdU⁺ cells was reproducibly lower in F0AI than control testes (Fig. 5E, I). This suggested that meiosis progression is normal but less efficient in F0AI testes. The normal meiotic progression was confirmed by counting the tubules that contained germ cells at various stages of prophase I, after incubation with an anti- γ H2aX antibody (marker of double-strand DNA breaks occurring in prophase I that shows a dynamic distribution through meiotic I prophase) (Supplemental Fig. S2B). Although meiotic prophase I was not significantly delayed in F0AI testes between 8 and 30 dpp, the percentage of tubules containing rSpds at 21 dpp and eSpds at 30 dpp was significantly lower in F0AI testes than controls (Fig. 5F). Similarly, rSpd number per tubule was significantly lower in F0AI testes compared to controls at these stages (Supplemental Fig. S1D, E). These defects were observed also in F1WT-AI, F1AI-WT, and F1AI-AI animals (Fig. 5G, H). These results suggest that spermatogenesis is less efficient in *in utero*-exposed animals (F0) and also in their offspring (F1), although in F1 animals the SpgA number was back to normal in late postnatal

stages, and consequently sperm count was not significantly affected.

Delayed Sertoli-cell maturation could contribute to effect on sperm count

Comparison of the transcriptome profiles of *in utero*-exposed 13.5 dpc testes and controls (Fig. 3A, B and Supplemental Tables S1 and S3) and the decrease in sperm count in F0 animals (Fig. 4B) suggested that fetal and postnatal Sertoli-cell function could also be affected. Postnatal Sertoli-cell maturation plays a central role in supporting spermatogenesis (5, 67). FACS analysis after labeling with an anti-Sox9 antibody showed that the total number of Sertoli cells was significantly decreased in F0AI testes compared to controls only at 8 dpp, but not at 14, 21, and 30 dpp (Supplemental Fig. S3A). However, Sertoli-cell proliferation (Sox9⁺EdU⁺ cells) was not affected even at 8 dpp (Supplemental Fig. S3B). Moreover, the number of Sox9⁺ cells per tubule was comparable in F0AI and control testes at all stages (Supplemental Fig. S3C). The expression of the *anti-Müllerian hormone* (*Amh*) gene, which encodes the marker of immature Sertoli cells (*Amh*) (67), remained higher in F0AI testes than in controls around puberty (Fig. 6A). Conversely, expression of *cell cycle inhibitor Cdkn1b* and *androgen receptor* (*Ar*), 2 markers of Sertoli-cell maturation (67), was significantly lower in F0AI testes than in controls at 8 and 14 dpp (Fig. 6B). Moreover, at 30 dpp, *Amh* expression was fully down-regulated in control testes, but not in F0AI and F1 testes (Fig. 6B). During Sertoli-cell maturation, the nucleus relocates from the center to the basal compartment of seminiferous tubules (67, 68). At 8 dpp, Sox9⁺ nuclei were not yet at the base of immature Sertoli cells in the exposed F0AI, similar to control testes (Fig. 6C). In the control testes, Sertoli-cell nuclei migrated to the basal position between 14 dpp (puberty) and 21 dpp (Fig. 6C). Conversely, in F0AI testes, Sox9⁺ Sertoli-cell nuclei were not yet in the basal compartment at 14 and 21 dpp. At 30 dpp, few disorganized Sertoli cells and some tubules containing centrally located Sertoli-cell clusters could be observed in F0AI testes (Fig. 6C), suggesting a delay in Sertoli-cell maturation during puberty. Therefore, the reduced sperm count observed in F0AI animals could be caused by a reduction of the SpgA pool and a delay in Sertoli-cell

Figure 5. SpgA pool is affected in F0 animals after birth but not in their F1 offspring. *A, B*) Mvh^+ (*A*) and $Lin28a^+$ (*B*) cells in 8, 14, 21, and 30 dpp control and F0AI testes were quantified by flow cytometric analysis of dissociated testis cells ($n = 3$ experiments). *C, D*) Number of $Lin28a^+$ cells/tubule in 8, 14, 21, and 30 dpp control and F0AI testes (*C*) and in 8 and 14 dpp control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI testes (*D*). Values are means $\pm$ SEM for $n = 4-6$ gonads/group; $n > 200$ counted cells group. *E*) Coimmunofluorescence analysis of Sox9 (red) and EdU (green) on 8, 14, 21, and 30 dpp control and F0AI testes. Arrows indicate EdU^+ germ cells, spermatocytes (14 dpp), rSpds (21 dpp), and eSpds (30 dpp). Scale bar, 50 μ m. *F*) Distribution of meiotic prophase I substages (leptotene, pachytene, and diplotene) was identified by γ H2AX staining distribution and presence of rSpds and eSpds identified with Hoechst and PAS staining. Number of rSpds and eSpds is decreased in 21 and 30 dpp F0AI animals, respectively. Data are presented as means $\pm$ SEM, $n = 6$ testes/group; $n = 100-200$ counted tubules. *G, H*) Number of rSpds/tubule (*G*) and percentage of eSpd-containing tubules (*H*) were determined in 30 dpp testes from control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI animals. Values are means $\pm$ SEM for $n = 5-8$ gonads in each group and $n = 80-120$ counted tubules. *I*) Number of EdU^+ cells/tubule in 8 (spermatogonia), 14 (spermatocyte), 21 (rSpds), and 30 dpp (eSpds) control and F0AI testes, relative to (*E*). Values are means $\pm$ SEM for $n = 3-5$ gonads in each group ($n = 30-50$ counted tubules). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$.

maturation. Finally, the percentage of basal Sox9⁺ cells in F1WT-AI, F1AI-WT, and F1AI-AI testes at 21 and 30 dpp remained significantly lower compared to controls, with values comparable to those of F0AI testes (Fig. 6D). These results suggest an intergenerational effect on Sertoli-cell maturation after *in utero* exposure of the mother or/and the father; however, this effect is not sufficient to significantly affect sperm production in F1 adults.

Spermiogenesis is impaired in adult F1AI-AI testes

The reduced motility of Spz in F1AI-AI animals and the presence of abnormal spermatids in the lumen of F1AI-AI tubules (Fig. 4F) could be the consequence of impaired spermiogenesis, which normally leads to the final

maturation of eSpds into Spz (69). At onset of spermiogenesis, deposition of the histone mark H4Kacetyl (K5-K8) is a prerequisite for histone replacement by transitional proteins (Tnp1/2) and then by protamines (Prm1/2) to facilitate chromatin condensation and compaction (69). In 30 dpp F0AI testes, expression of *Tnp1*, *Tnp2* and *Prm1*, *Prm2* was significantly up-regulated compared to controls (Fig. 7A). Moreover, immunofluorescence analysis showed that step 9 to 10 and step 12 spermatids (late differentiating rSpds) were strongly and similarly labeled by an anti-H4Kacetyl (K5-K8) antibody (70), in 30 dpp and 3 mo old control, F0AI, and F1AI-AI testes (Fig. 7B, C). Conversely, 100% of step 1 to 8 spermatids (early differentiating rSpds) were labeled in 30 dpp F1AI-AI (Fig. 7B) and in 3 mo old F0AI and F1AI-AI (Fig. 7C) testes, but very weakly in 30 dpp and 3 mo old control, in 30 dpp F0AI (Fig. 7B, C), and in F1WT-AI and F1AI-WT (unpublished observations)

Figure 6. Sertoli-cell maturation is delayed in F0 and F1 animals after birth. *A*) qRT-PCR analysis of *Amh*, *Cdkn1b*, and androgen receptor (*Ar*) expression levels normalized to *18S* expression and presented as means $\pm$ SEM ($n = 3$ independent samples). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. *B*) Immunofluorescence analysis of *Amh* expression (red, arrows; marker of immature Sertoli cells) in 30 dpp control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI testes. HST, Hoechst dye (blue). *C*) Immunofluorescence analysis of Sox9 expression (red) and Hoechst staining (HST, blue) on 8, 14, 21, and 30 dpp control and F0AI testes. Enlarged panels show details of representative localization of Sox9⁺ Sertoli cells. Arrows and arrowheads indicate abnormal and normal basal position of Sertoli cells, respectively. *D*) Quantification of basal Sox9⁺ Sertoli cells in 21 and 30 dpp testes from control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI testes; data are presented as percentage of normally localized cells at base of tubules relative to total number of Sox9⁺ cells. Values are means $\pm$ SEM ($n = 6-9$ gonads in each group and $n > 50$ counted Sox9⁺ cells/testis). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$. Scale bars, 50 μ m (B, C).

testes. This suggests that the histone removal/replacement could be advanced in F0AI and F1 testes when both parents have been exposed *in utero*. On the other hand, the macroscopic shape of Spz, the acrosome, and the global characteristics of the sperm head were normal in F0 and F1 animals (Fig. 8A), suggesting that the Spz were correctly formed. The assembly of the sperm flagellum involves the formation of the microtubule-based axoneme, the central component of the flagellum (71). Microtubules in the sperm axoneme are extensively posttranslationally modified, including by acetylation (72). Immunofluorescence analysis with anti- α -tubulin and anti-acetylated α -tubulin antibodies of Spz in caudal sperm spreads showed α -tubulin expression mainly in the manchette, at the midpiece of the flagellum, and at the end piece of the flagellum in control and also F0AI and F1AI-AI samples (Fig. 8B). Acetylated α -tubulin expression pattern in control and F0AI Spz was similar to that of α -tubulin; however, tubulin hyperacetylation

throughout the flagellum was observed in F1AI-AI (Fig. 8C) but not in F1WT-AI and F1AI-WT sperm (unpublished observations), suggesting abnormal acetylation in the axoneme/flagellum that could affect sperm motility.

DISCUSSION

In this study, we described the effects in postnatal mouse testis and the possible underlying cellular mechanisms induced by *in utero* exposure to human-relevant doses of the widely used antiinflammatory (ASA and IBU) and analgesic (APAP) drugs during the sex determination period. Specifically, *in utero* exposure to the APAP + IBU combination leads to sperm count reduction in F0 adult mice and also to sperm motility defects in their offspring (F1 mice) when both parents have been exposed. We provide evidence that *in utero* exposure to these drugs at

Figure 7. Histone replacement is affected in nonexposed F1 mice. **A**) qRT-PCR quantification of genes encoding transition proteins Tnp1 and Tnp2 and protamines Prm1 and Prm2 normalized to 18S expression in 30 dpp control (C) and F0AI testes; data are presented as means $\pm$ SEM ($n = 3$ independent samples). $**P < 0.01$, $****P < 0.0001$. **B, C**) Distribution of histone H4 K5-8 acetylation (H4K5-8ac) in 30 dpp (**B**) and 3 mo old (**C**) testes by immunofluorescence analysis with anti-H4K5-8ac antibody (red). Nuclei were stained with Hoechst (blue). Roman numerals refer to stages of seminiferous epithelium cycle, and spermatid differentiation steps are indicated. White arrows indicate H4K5-8ac staining on steps 1 to 8 spermatids (Spds), and white arrowhead indicates staining in differentiating spermatogonia. Scale bars, 25 μ m.

the time of sex determination leads to early differentiation and decreased proliferation of male embryonic germ cells, early 5-mC, and increased ECM protein deposition in 13.5 dpc APAP + IBU exposed testes. These changes affect the

normal differentiation and maturation of postnatal somatic and germ-cell lineages. We also showed that in mouse embryonic testes, APAP + IBU *in utero* exposure affects at least 3 PGs—PGD₂ (31), PGE₂, and PGI₂—thus

Figure 8. Posttranslational tubulin acetylation in sperm is affected in nonexposed F1 adult mice. **A**) Macroscopic analysis of control, F0AI, F1WT-AI, F1AI-WT, and F1AI-AI caudal sperm. Sperm spreads were stained with Hoechst (DNA, blue); immunofluorescent and bright-field images are superposed. **B, C**) Localization of α -tubulin (α -Tub) (**B**) and acetylated α -tubulin (Ac α -Tub) (**C**) in control, F0AI, and F1AI-AI caudal sperm by immunofluorescence with anti- α -Tub and anti-Ac α -Tub antibodies (green) with Hoechst (HST, blue). Corresponding bright-field images are shown at right. Scale bars, 200 μ m.

confirming that PGs are involved in testis development, maturation, and function (31, 32).

These results suggest that exposure to this widely used drug combination involves complex mechanisms and might affect the genome and/or gene regulation in developing germ cells, as has already been described for environmental chemicals in rodents and humans (2). Whereas exposure to single drugs only affect cell proliferation inducing early embryonic germ-cell mitotic arrest, as already described (26), we found that cell proliferation and differentiation in 13.5 dpc testes are affected by APAP + IBU exposure, as indicated by the up-regulation of the male sexual differentiation markers *Sox9* and *Dnmt3L* and the decreased expression of the pluripotency gene *Sox2*. These results suggest that APAP and IBU may interact with each other to affect cell differentiation in the embryonic testis. Exposure between 10.5 and 13.5 dpc, the time of the genome-wide reduction in DNA methylation of the primordial germ cell (PGC) genome (59, 73), might shield some regions from complete DNA methylation erasure. Moreover, *Dnmt3L* up-regulation might alter the correct DNA methylation reacquisition of some regions (nonimprinted or imprinted loci) within the developing PGC genome. It might also interfere with the remodeling of histone modifications that takes place during PGC epigenetic reprogramming between 11.5 and 12.5 dpc (74). Because chromatin remodeling of human fetal germ cells occurs during the first trimester of pregnancy (75), our results have a clear relevance to human reproductive health. Concomitantly, increased *Sox9* expression in testes from 13.5 dpc embryos exposed *in utero* to APAP + IBU, which could be the result of the antagonist effect of PGE₂ and PGI₂ *vs.* PGD₂, could lead to the up-regulation of genes that encode ECM proteins (61, 62). This could increase storage of glycoproteins within the testis cords that are involved in the maintenance of *Sox9* expression (76) and that are required for testis cord basal membrane integrity (77). Alteration of embryonic Sertoli-cell function, together with the early epigenetic reprogramming and the down-regulation of pluripotency genes, might be responsible for the early differentiation of the male germ line after *in utero* APAP + IBU exposure.

The alteration of fetal gonocyte development kinetics does not compromise the subsequent reactivation of spermatogonia proliferation and postnatal development during puberty, as indicated by the presence of apparently normal germ-cell populations in postnatal and adult testes, and does not affect the reproductive ability of adult *in utero*-exposed animals. However, sperm count in F0AI adults was significantly reduced, suggesting impaired spermatogenesis. This might be the dual consequence of the reduced SpgA pool and the delayed Sertoli-cell maturation in postnatal testes because mature Sertoli cells play a central role in supporting spermatogenesis (5, 67). The normal anogenital distance index in postnatal F0 animals suggests a normal level of fetal androgen exposure and consequently a normal function of fetal Leydig cells. However, impaired postnatal Sertoli-cell differentiation could have an impact on ALC development (78). ALCs could be altered or insufficiently activated during and after the pubertal period. Indeed, the low testosterone pituitary

back-regulation that increases the gonadotropin concentration could not be enough to increase testosterone levels in F0AI animals but sufficient to stimulate spermatogenesis, because a mild decreased sperm count (19%) was found in these mice. On the other hand, high follicle-stimulating hormone level could lead to a compensatory effect on sperm production in an androgen-independent manner (79, 80). Also, the high LH stimulation that might result could strongly promote ALC proliferation, resulting in hyperplasia. Overexpression of some steroidogenic genes and decreased production of testosterone in F0AI testes are indicative of compensated ALC failure that might originate from an impaired proliferation/differentiation process of ALCs. In humans, the compensated dysfunction of Leydig cells has been associated with lower sperm quality (81). Similarly, hyperplasia of Leydig cells has already been described in rodents after *in utero* exposure to phthalates (63, 82).

These defects could be caused by changes in germ-cell/somatic transcriptomes and/or epigenome profiles after *in utero* exposure (Fig. 9). These changes could alter the regulation of developmental genes, both directly by affecting the control of the epigenetic landscape of postnatal germ cells and their progression through spermatogenesis (83–85) and indirectly through the actions on Sertoli and Leydig precursor cells (5) (Fig. 9). Similar effects on gonad development and reproductive health of F0 adult female rodents have been described after exposure to high doses of APAP or indomethacin (26, 27). However, fertility of adult male rats (26) and sperm parameters in mice (25) were not altered by exposure *in utero* to these drugs.

Our findings show that Sertoli-cell maturation is delayed and that Leydig cells are hyperplastic in F1 testes when at least one of the F0 parents was exposed *in utero*. This suggests that some modifications caused by *in utero* exposure to APAP and IBU are inherited through the male or female germ line, and that these modifications are stable after fertilization (Fig. 9). However, in F1WT-AI, F1AI-WT, and F1AI-AI testes, the SpgA pool is compensated and production of testosterone is normal, leading to a normal sperm count. Conversely, sperm motility, which is one of the most important predictors of fertilizing ability in humans, was affected only in F1AI-AI animals (both parents exposed *in utero*), suggesting that the 2 parental genomes exposed *in utero* need to interact, generating this defect as a consequence of exposure-induced epigenetic modifications (Fig. 9). These modifications could lead to impaired spermiogenesis (69) and possibly sperm motility in F1AI-AI animals. Alterations in germ-cell epigenetic markers (86, 87) and in the sperm epigenome have already been linked to defects in spermiogenesis and to various types of male infertility (88, 89) in rats (90) and in men with poor sperm motility (91). Modified axonemes and incomplete acrosomes of Spz were observed after postnatal APAP administration in rats, possibly affecting sperm movement (92).

In summary, here we show that in the mouse, APAP and IBU have an important impact on the germ-cell development and maturation program in embryonic testis and can induce intergenerational effects that can be inherited, possibly through epigenetic mechanisms (Fig. 9). This suggests

Figure 9. Summary of experimental design and phenotypes of F0 and F1 mice after *in utero* exposure to APAP and IBU. Pregnant mice were treated with APAP and IBU from 10.5 to 13.5 dpc. Exposure may directly affect F0 soma and F0 germ line of embryos, and indirectly F1 germ cells as well as F1 soma that develop from F0 germ cells. Red, blue, and green stars indicate modifications of germ, Sertoli, and Leydig cells, respectively. Light and dark colors indicate phenotypes transmitted through maternal and paternal effect, respectively.

that their use by pregnant women, particularly during the critical period of sex determination, could lead to adverse effects in humans (93). Additional studies are now required to evaluate whether these effects might be transmitted across several generations or might be reversible. Also, other studies will identify the modifications of the PGC epigenome modifications that might be inherited and lead to inter- or transgenerational effects, as shown after exposure to various endocrine disruptors (60). The sensitivity toward these drugs may differ in humans compared to rodents. Nevertheless, because of the widespread use of analgesic drugs for various indications in pregnancy, our results suggest that the safe therapeutic doses of these molecules should be reevaluated and measures taken to limit their intake during the first trimester of pregnancy. Also, our mouse model of *in utero* NSAID/APAP exposure provides a valuable tool for investigating their effect, and also of other drugs and environmental chemicals, on human male reproduction, sperm quality, and fertility. ^[F]

ACKNOWLEDGMENTS

The authors thank J. Cau, M.-P. Blanchard, and A. Sarrazin (Institute of Human Genetics Imaging Facility, MRI Montpellier, University of Montpellier) for their help in microscopy and

FACS analysis, and N. Pirot and F. Bernex (Réseau d'Histologie de Montpellier, University of Montpellier) for advice on histology staining and analysis. The authors thank the staff of the Institute of Human Genetics Animal Care Facility. The authors also thank N. di Clemente (University of Paris Diderot) for providing antibody against Amh/Mis, and Véronique Vidal and Yannick Lopez (Helixio, groupe Hybrigenics, Saint-Beauzire, France) for providing RNA-seq data. The authors are grateful to D. Giorgi for critical reading of the report and P. Clair (qPCR platform, Biocampus Montpellier) for advice on statistical analysis. This study was supported by the Centre National de la Recherche Scientifique (CNRS), the University of Montpellier, and by National Agency for the Safety of Health Products (ANSM) Grant 2013-041. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Rossitto, A. Pruvost, E. Sellem, P. Philibert, and B. Boizet-Bonhoure designed the research; M. Rossitto, C. Marchive, A. Pruvost, E. Sellem, A. Ghetas, S. Badiou, T. Sutra, P. Philibert, and B. Boizet-Bonhoure performed the experiments; M. Rossitto, C. Marchive, A. Pruvost, E. Sellem, T. Sutra, F. Poulat, P. Philibert, and B. Boizet-Bonhoure analyzed the data; M. Rossitto, P. Philibert, and B. Boizet-Bonhoure wrote the manuscript; and all authors contributed to discussions.

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