Paracetamol, Aspirin, and Indomethacin Induce Endocrine Disturbances in the Human Fetal Testis Capable of Interfering With Testicular Descent

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Context: Masculinization depends on the fetal testis. Exposure of the human fetus during pregnancy to paracetamol and/or to other mild analgesics is associated with an increased risk of cryptorchidism.

Objective: We aimed to determine whether mild analgesics disrupted the morphology and endocrine function of the human testis.

Design: We used an in vitro system based on the culture of human fetal testes exposed or not to paracetamol, its metabolite N-(4-hydroxyphenyl)-arachidonoylethanolamide (AM404), aspirin, indomethacin, and ketoconazole at 10^{-4} to 10^{-7} M.

Setting: The study was conducted at the University of Rennes I.

Patients/Participants: Human fetal testes were from pregnant women after induced abortion, between 7 and 12 weeks of gestation (GW).

Main Outcome Measures: Testosterone (RIA), anti-Müllerian hormone (ELISA), insulin-like factor 3 (RIA), and prostaglandin (PG) D_2 and PGE_2 (ELISA) were assayed in the medium. Testicular cells were counted using histology and image analysis. The possible nuclear receptor–mediated activities of the analgesics were investigated using reporter cell lines expressing estrogen, androgen, and peroxisome proliferator–activated γ receptors.

Results: Indomethacin and aspirin stimulated testosterone production, particularly by the younger testes (8–9 GW vs 10–12 GW). Paracetamol, AM404, and ketoconazole decreased insulin-like factor 3 levels. Aspirin stimulated whereas ketoconazole inhibited AMH production. PGE_2 levels were inhibited by paracetamol and aspirin in the 7 to 12 GW testes and by indomethacin but only in 7 to 9.86 GW testes. The inhibitory trends seen for PGD_2 were not statistically significant.

Conclusions: Analgesics at concentrations relevant to human exposure cause endocrine disturbances in the fetal testis. We suggest that the fetal human testis displays slight critical age windows for sensitivity to direct exposure to aspirin, indomethacin, and paracetamol. The analgesic-induced inhibition of INSL3 may be the mechanism by which analgesics increase the risk of cryptorchidism. Greater caution is required concerning consumption of analgesics during pregnancy. (*J Clin Endocrinol Metab* **98: E1757–E1767, 2013**)

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Abbreviations: AMH, anti-Müllerian hormone; AR, androgen receptor; COUP-TF II, chicken ovalbumin upstream promoter transcription factor 2; CV, coefficient of variation; ED, endocrine disruptor; ER, estrogen receptor; GW, weeks of gestation; INSL3, insulin-like factor 3; NSAID, nonsteroid anti-inflammatory drug; PG, prostaglandin; PPARγ, peroxisome proliferator-activated receptor γ.

The differentiation of the gonads in humans into male or female begins around 6 weeks of gestation (GW). The sex-determining region Y (*SRY*), a Y-chromosome-linked gene, triggers expression of a number of downstream genes that control the differentiation of testis-specific cell types, their migration, and the formation of the fetal testis cords (1). Once differentiated, the testis secretes a number of hormones that control the development and differentiation of the male genital tract (2) and are in order of appearance (1) the anti-Müllerian hormone (AMH) produced by Sertoli cells, which acts on the Müllerian ducts to induce their regression (3), (2) testosterone produced by fetal Leydig cells, which ensures the differentiation of Wolffian ducts (3) and the terminal phase of testis descent and is involved in body-wide masculinization (4), and (3) the insulin-like factor 3 (INSL3), produced by differentiated fetal Leydig cells and involved in the transabdominal phase of testis descent (5–8). In addition, prostaglandins are believed to be involved in the differentiation of the testis and the male genital tract (9, 10). The development of the male reproductive system thus requires the action and interplay of different hormones and is therefore a priori highly susceptible to endocrine disruption.

Cryptorchidism (undescended testes) is the most common congenital malformation in the human male and affects 2% to 9% of full-term children (8). Its incidence was reported to have increased in industrialized countries over recent decades (11, 12). Undescended testis is the main risk factor for infertility and testis cancer (13, 14), the incidence of the latter cancer also being on the rise (12). Endocrine disruptors, alone or in combination with particular genetic susceptibilities, can trigger alteration of fetal testicular physiology, leading to cryptorchidism (12, 15, 16).

Nonsteroidal anti-inflammatory drugs (NSAIDs) and paracetamol/acetaminophen (paracetamol in the following) are among the most widely used drugs in industrialized countries, paracetamol being present in the microgram to milligram range in the urine of all individuals investigated (17). These pharmaceutical agents have recently joined the growing list of potential endocrine disruptors (EDs) in humans (18, 19). Two independent Danish studies and one Dutch epidemiological study recently reported that exposure to paracetamol alone or in combination with NSAIDs during both the first and the second trimesters of gestation is associated with an increased risk of cryptorchidism (19–21). These findings are consistent with those of an earlier American study showing an association between the use of analgesics during pregnancy and an increased risk of cryptorchidism (22). A recent French epidemiological study reported a similar trend with paracetamol (23). Experiments with rats revealed that intrauterine exposure to paracetamol reduces the anogenital distance in the male offspring, anogenital distance being a very sensitive marker of intrauterine exposure to androgens (19, 24). Experiments in vitro demonstrated that paracetamol, aspirin/acetyl salicylic acid (aspirin in the following) and indomethacin directly inhibit testosterone production by the rat fetal testes (19, 25). Like other EDs, mild analgesics were also found to inhibit testicular prostaglandin synthesis, thus diversifying the possible pathways by which EDs may exert their negative effects on the development of the male genital tract (19, 25, 26).

For ethical reasons, investigation on the direct effects of analgesics on the human fetal genitourinary tract is not possible; therefore, in the present study we set up an organotypic culture system based on human fetal testis cultured for 1 to 3 days to investigate whether mild analgesics have the ability to disrupt the hormonal homeostasis within the human fetal testis.

Materials and Methods

Collection of human samples

First trimester human fetal testes (7–12 GW) were obtained from pregnant women after legally induced abortion in Rennes University Hospital (Rennes, France); all women received information and gave verbal consent in accordance with national guidelines (Agence de la Biomédecine, authorization PFS09–011) and was approved by the local ethics committee of Rennes (11–48). The termination of pregnancy in all cases was induced by treatment with mifepristone (Mifegyne) followed 48 hours later by misoprostol (Cytotec) treatment and subsequently by aspiration. None of the terminations was motivated by fetal abnormality. The testes were recovered from the aspiration product under a binocular microscope (Olympus SZX7) and immediately placed in cold PBS. A total of 62 fetuses were used in this study.

Organ cultures

The testes recovered were cut into approximately 1 mm³ pieces. The youngest testes (7–9 GW) were cut into 4 pieces and 2 pieces corresponding to half a testis in the length were cultivated in each well of 24-well companion plates (Becton-Dickinson). In this case, 4 culture conditions including 1 control were

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designed. The oldest testes (10–12 GW) were cut into 8 pieces each, and each explant was cultivated separately. In this case, 1 testis was prepared according to an experimental design so that at least 2 solvent control conditions were included per fetus. Explants were cultured in cell culture inserts (0.4-μm pores) placed in the 24-well companion plates. Each well was filled with 350 μL of phenol red–free medium 199 (Invitrogen Life Technologies) supplemented with 50 $\mu g/mL$ gentamicin and 2.5 $\mu g/mL$ Fungizone (Sigma-Aldrich). To sustain steroidogenic responsiveness, human chorionic gonadotrophin (Sigma-Aldrich) was added to a final concentration of 0.1 IU/mL (27). Cultures were incubated at 37°C for 96 hours under a humidified atmosphere of 95% air and 5% CO_2. The medium was removed every 24 hours, divided into at least 2 aliquots that were immediately snap-frozen on dry ice and stored at −80°C. Explants were incubated in medium 199 and human chorionic gonadotrophin for 24 hours without any other drug; this served as a baseline for hormone production allowing normalization of explant secretory capacity before the addition of analgesics. After 24 hours of culture, the samples were exposed to treatments by adding to the medium either N-(4-hydroxyphenyl)-arachidonoylethanolamide (AM404; Ascent Scientific), which is a metabolite of paracetamol (28), paracetamol, aspirin, indomethacin, or ketoconazole (Sigma-Aldrich), an antifungal drug with antiandrogenic effects on the testis (18, 29). Estimating the doses of the analgesics to which the human fetal testes in culture should be exposed was not an obvious task when considering that, for example, in the case of an exposure during pregnancy the maximal serum concentration in the mother is likely to be higher than the dose that reaches the testes of the fetuses. Furthermore, AM404 has never been assayed in the blood. With these reserves in mind we considered the following: (1) Studies on paracetamol metabolism and pharmacokinetics in pregnant and nonpregnant women show that a standard dose (1 g) resulted in a serum concentration peaking at 20.8 $\mu g/mL$ (1.37×10^{-4} M) 48 minutes after intake (30). (2) Paracetamol can be metabolized by placenta and fetal liver and can pass through the placenta such that there is no difference in serum concentrations between the mother and fetus after an oral intake of 1 g (31). (3) A 600-mg dose of aspirin, taken orally, leads to a maximal serum concentration of 2×10^{-4} M in adult men (32). Therefore, we chose to screen for effects of these drugs at a test concentration of 10^{-5} M, which according to these exposure values, is in the range of expected circulating levels after routine use of these drugs; we also extended our investigations to concentrations of 10^{-4}, 10^{-6}, and 10^{-7} M to assess dose-response effects. Drugs were all diluted in DMSO (Sigma-Aldrich), and DMSO was used as a solvent control.

Immunohistochemistry and cell counting

Each piece of cultured testis was removed from the culture insert and fixed in 4% paraformaldehyde for 1 hour and then washed with PBS. The whole explants were then embedded in paraffin and cut into 5-μm-thick sections. Each fifth section was used for immunohistochemical analysis with cell-specific labeling: samples were dewaxed and rehydrated and processed for immunohistochemical staining using a standardized indirect streptavidin-biotin peroxidase method. AMH immunostaining required antigen retrieval, so the sections were treated with preheated 10 mM citrate buffer (pH 6.0) at 80°C for 40 minutes and cooled at room temperature. For all immunostaining, sections

were blocked for 1 hour at room temperature with 4% BSA in PBS and then incubated overnight at 4°C with the primary antibody diluted in Dako antibody diluent (Dako Cytomation). The primary antibodies used were mouse monoclonal antibodies to AMH (MCA2246T; AbD Serotec; diluted 1:100) and to chicken ovalbumin upstream promoter transcription factor 2 (COUP-TF II/NR2F2) (PP-H7147; Perseus Proteomics, R&D Systems; diluted 1:100), and rabbit polyclonal antibodies to INSL3 (HPA028615; Sigma-Aldrich; diluted 1:100). The secondary antibodies were goat biotinylated anti-rabbit antibody (E0432; Dako Cytomation; diluted 1:500 for INSL3) and biotinylated rabbit anti-mouse antibody (E0464; Dako Cytomation; diluted 1:500 for AMH and COUP-TF II). Sections were developed with streptavidin-horseradish peroxidase (Vectastain ABC kit; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and counterstained with hematoxylin. Stained sections were examined and photographed under the light microscope (Olympus BX51). Cell counting was performed using the semiautomatic counting CAST grid image analysis software (Olympus) based on stereological principles. Germ cells and Sertoli cells were identified and counted as intratubular AMH-negative and AMH-positive cells, respectively (33). Mesenchymal cells of the interstitial tissue were identified by their COUP-TF II staining (34). Cells were counted in at least 5 sections, and cells counts are reported as numbers per unit surface area of the section.

Hormone measurements

Hormone levels were measured in culture media of testis explants. Day 0 (the first 24 hours) served as the baseline value for normalization of hormone production per piece of testis after 72 hours of culture with the drugs. For drug effects on testosterone and INSL3 production, measurements were repeated at 24, 48, and 72 hours for each explant. For all hormone measurements, each sample was assayed in duplicate. Testosterone was measured by RIA according to the kit manufacturer's instructions (intra-assay coefficient of variation [CV] of 7.5%−14.8% and interassay CV of 6.9%−15%; testosterone direct RIA; Immunotech). Fragments of 8 GW testis were the youngest to produce sufficient testosterone to be detected. INSL3 was assayed by using the INSL3/RLF (human) RIA kit (intra-assay CV of 9.86%−41.72%; Phoenix Pharmaceuticals, Phoenix Europe GmbH) with samples obtained after 10 GW. AMH was assayed by an ELISA method from 8 GW on (intra-assay CV of 3.2%−12.3% and interassay CV of 5.8−14.2%, AMH/MIS kit; Immunotech). Prostaglandin (PG) D_2 and PGE_2 were assayed by an ELISA method (intra-assay CV of 8%−15% and interassay CV of 10%−17% and intra-assay CV of 3.7%−30.4% and interassay CV of 6.4%−35%, respectively; Prostaglandin D_2-MOX EIA Kit and Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical Company) with samples from 7 GW on.

Assessment of nuclear receptor–mediated agonist or antagonist activities

The stably transfected luciferase reporter cells HGELN–estrogen receptor α (ERα), HGELN–androgen receptor (AR) (ERα DNA–binding domain) and HGELN-GAL4 (DNA–binding domain)–peroxisome proliferator–activated receptor γ (PPARγ) (ligand-binding domain) were described previously (35). To measure ERα, AR, and PPARγ activity, the reporter cells were seeded at a density of 40,000 cells/well in 96-well white opaque

tissue culture plates (Greiner; Dutscher). Analgesics and reference compounds (estradiol [Sigma-Aldrich] and ICI172,780 for ERα [Tocris]), R1881 [Roche Diagnostics] and hydroxy flutamide [Tocris] for AR, and rosiglitazone [Tocris] for PPARγ) were added 8 hours later, and cells were incubated for 16 hours. At the end of incubation, culture medium was replaced by medium containing 0.3 mM luciferin. Luciferase activity was measured for 2 seconds in intact living cells using a MicroBeta Wallac luminometer (PerkinElmer). Tests were performed in quadruplicate in at least 3 independent experiments, and data was expressed as means ± SD. Dose-response curves were fitted using the sigmoid dose-response function of a graphics and statistics software package (Graph-Pad Prism, version 4, 2003; Graph-Pad Software Inc).

Statistical analysis

We assessed the potential impact of the treatments on cell counts using nonparametric Kruskall-Wallis test. For quantitative measurements of hormones, similar nonparametric approach was used when normality distribution was not hold. These statistical tests were performed using the SigmaStat 2.0 software package. For hormone measurements normally distributed, we performed analyses of variance including the treatment as explanatory variable and a random testis effect. For testosterone and INSL3 data, which involve both treatment and culture time variables, we additionally accounted for the repeated measurements (24, 48, and 72 hours) within each treatment, by using an unstructured covariance matrix in the models. Models included an interaction term between time and treatment to allow, for instance, estimation of possible differences in changes in hormone levels between treatments and controls within each culture time period. For each of the paracetamol and aspirin treatments, a dose-response relationship was assessed for testosterone and INSL3 measurements at 72 hours using random ANOVA. The dose was transformed into a categorical variable (from 1 for the lowest dose to 5 for the highest dose) and was introduced into the models as a continuous variable. The corresponding slope represents the change in hormone secretion for each supplementary dose unit ($-\log 10$–transformed). A natural logarithm transformation was used for INSL3 to normalize the data. Values of $P < .05$ were considered to indicate statistical significance. SAS software was used for these calculations (SAS/STAT version 9.2; SAS Institute Inc).

Results

Analgesics and the fetal testis gross anatomy

No change in the testis architecture was observed with the analgesic treatments, whereas ketoconazole rendered unrecognizable the boundaries of the testis cords (Figure 1A). Cell proliferation rates did not differ within the testicular cords between the control and exposed testes (Figure 1B). None of the mild analgesics significantly modified either the number of germ cells or the germ to Sertoli cell ratio (Figure 1, C and D).

Analgesics and testosterone production

Ketoconazole markedly reduced testosterone levels in a time-dependent manner (Figure 2A). In contrast, indomethacin stimulated testosterone production (+16% at 48 hours and +32% at 72 hours) (Figure 2B). We found that the latter stimulatory effects were higher for 8 to 9.86 GW (+50%) (Figure 2B) than 10 to 12 GW (+29%) (Figure 2B) testes. Neither the paracetamol metabolite AM404 (10^{-5}M) nor paracetamol itself at 10^{-5} M (Figure 2B) or between 10^{-7} and 10^{-4}M in other experiments had any significant effect on testosterone production. A significant dose-response relationship was observed, indicating an increase in testosterone levels after 72 hours of exposure to aspirin at doses of 10^{-7} to 10^{-4} M of the youngest fetal testes (8–9.86 GW; slope $\beta = 0.04$, $P = .01$) (Figure 2C) but not in the older testes (10–12 GW) (Figure 2C), thus indicating the particular sensitivity of the fetal testes to aspirin during the first trimester of development. At 10^{-5} M, aspirin had no significant effects when all ages were pooled (Figure 2A). None of the treatments with analgesics significantly affected the number of interstitial cells (Figure 2D).

Nuclear receptor–mediated agonistic or antagonistic activities

Analgesics did not display either receptor-mediated androgenic/antiandrogenic (Figure 3, A and B) or estrogenic/antiestrogenic (Figure 3, C and D) activities. Indomethacin but not aspirin or paracetamol bound to and activated PPARγ (Figure 3, E and F).

Analgesics and INSL3 production

There was a consistent trend for lower INSL3 production after 48 and 72 hours of exposure to mild analgesics or ketoconazole at 10^{-5} M than in controls ($P < .0001$) (Figure 4A). In samples exposed to AM404, significant inhibition of INSL3 production was found after 24 hours ($P = .02$), 48 hours ($P = .009$), and 72 hours ($P = .005$). After 72 hours of exposure to 10^{-5} M paracetamol, INSL3 titers were lower than in controls ($P = .0543$). In addition, a significant dose-response relationship was observed (slope $\beta = -0.13$, $P = .02$), indicating a decrease in INSL3 production with an increasing dose of paracetamol from 10^{-7} to 10^{-4} M (Figure 4B). INSL3 being measurable only from 10 GW, the age range of the fetal testis (10–12 GW) used here was too narrow to allow identification of a possible critical window of sensitivity to mild analgesics. The counts of INSL3-positive cells after 72 hours of exposure to the drugs were not significantly different from control values (Figure 4C).
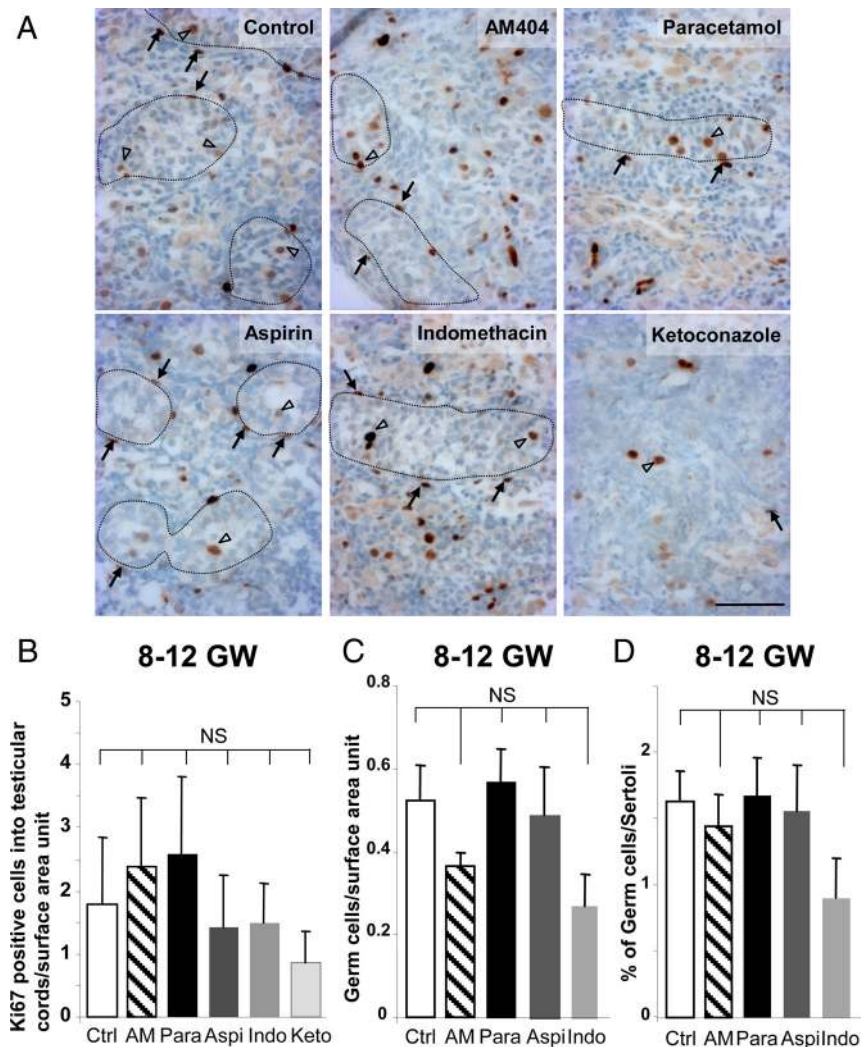
Figure 1. Analgesics and gross anatomy after culture of the human fetal testis. A, Representative immunostaining of Ki67 in cultured explants of an 11 GW human fetal testis. The 3,3′-diaminobenzidine tetrahydrochloride staining appears dark in all panels, and sections were counterstained with hematoxylin. Testis cords could be easily delineated in all treated explants except in the sections of explants that have been exposed to ketoconazole, in which the boundaries of the testicular cords appeared consistently blunted. Ki67-positive germ cells (arrowheads) and interstitial/peritubular cells (arrows) are indicated. Note that cytoplasmic staining is background staining. Scale bar corresponds to 100 μm. B, Cell proliferation rates were determined by counting Ki67-positive cells within the seminiferous tubules after culture of human fetal testis (n = 3–4 testes from 3–4 fetuses of 7–12 GW) in the presence of 10^{-5} M DMSO (solvent control [Ctrl]), AM404 (AM), paracetamol (Para), aspirin (Aspi), indomethacin (Indo), and ketoconazole (Keto). C and D, numbers of Sertoli cells (C) were determined by counting AMH-positive cells. The gonocyte rate (AMH-negative cells) and germ to Sertoli cell ratio (D) were determined by counting after culture of human fetal testis (n = 4–7 testes from 4–7 fetuses) in presence of molecules of interest. Data are presented as number of cells per surface area unit (defined as 0.01 μm²) or percentage of germ cells to Sertoli cells per surface area unit (0.01 μm²) (means ± SEM) based on 1 or 2 explants per treatment from 3 to 4 fetuses (Ki67) or 4 to 7 fetuses (germ and Sertoli cells). Kruskall-Wallis ANOVAs were performed. NS, not significant.

Analgesics and AMH production

Aspirin strongly stimulated AMH production by Sertoli cells (+71%, after a 72-hour exposure; $P = .02$) (Figure 5A). The consistent trends for AMH production to be increased by paracetamol (+40%) and indomethacin (+37%) were not significant. None of the analgesics sig-

nificantly affected the numbers of Sertoli cells (Figure 5B). Ketoconazole significantly inhibited AMH production (−65%), an observation confirmed in situ (Figure 5, A and C). No particular age window during which AMH production by human fetal testis was sensitive to the mild analgesics was found.

Analgesics and prostaglandin production

When all fetal ages were pooled (7–12 GW), exposure of the human fetal testis explants to paracetamol and aspirin led to a significant inhibition of PGE_2 production, whereas indomethacin had no effect (Figure 6A). The significant inhibitory effects of paracetamol were observed both in the youngest (7–9.86 GW) and oldest testes (10–12 GW) (Figure 6, B and C). Whereas aspirin and indomethacin significantly decreased PGE_2 production in the youngest testes (7–9.86 GW), this was not the case in the oldest ones (10–12 GW); the aspirin-induced inhibition (−31%) was, however, not significant ($P = .114$) (Figure 6, B and C). When the testes were exposed to the different analgesics, the trends for inhibition of PGD2 observed were never significant (Figure 6, D–F).

Discussion

Four independent studies have shown a significant association between the intake of paracetamol alone or in combination with NSAIDs during pregnancy and an increased risk of cryptorchidism in newborns (19–22). Two other cohorts, a Finnish one (19) and a French one (23), displayed equivalent trends, but these studies may have been statistically underpowered to find significant associations (19). For obvious ethical reasons, experiments involving experimental exposure of pregnant women to mild analgesics are impossible to undertake; therefore, the only possible way to investigate the origin of the deleterious effects of these drugs on testicular descent
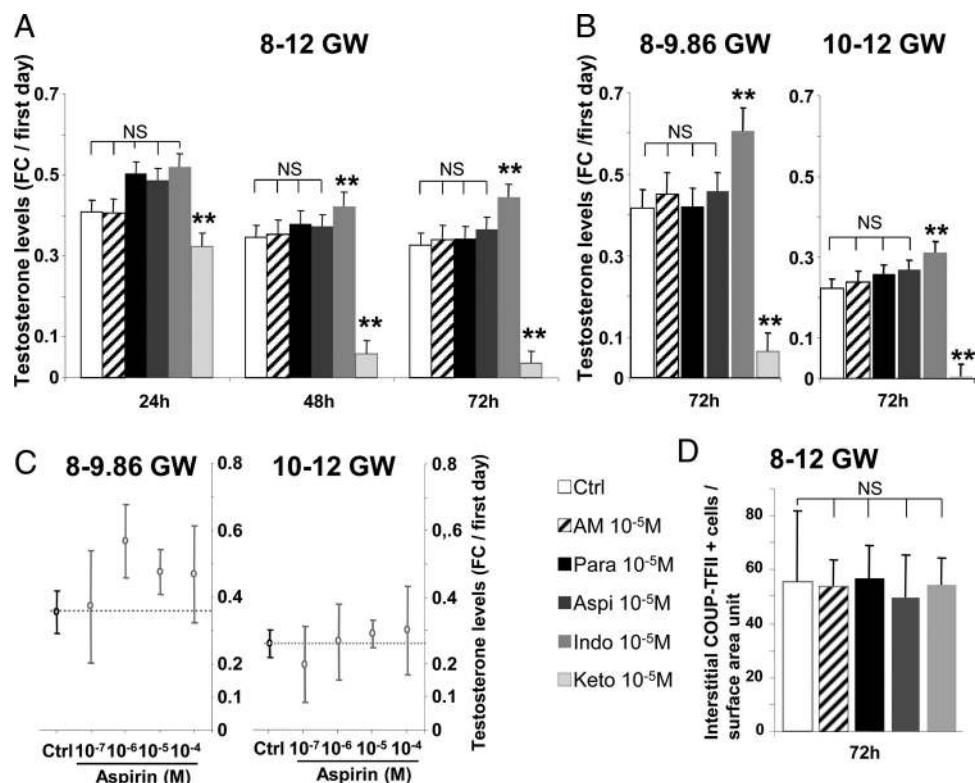
Figure 2. Analgesics and testosterone production after culture of 8 to 12 GW human fetal testes. A, Testosterone concentrations after culture of human fetal testes (8–12 GW) in the presence of DMSO (solvent control [Ctrl]) or 10^{-5} M AM404 (AM), paracetamol (Para), aspirin (Aspi), indomethacin (Indo), or ketoconazole (Keto). Results are expressed as fold change from the respective untreated first day of culture sample (FC/first day). Values are least squares means ± SE of 61 testes from 55 fetuses. Repeated-measures analyses of variance with a random embryo effect were performed using unstructured covariance matrices (**, $P < .01$). B, Effect of treatments on testosterone production after 72 hours of exposure subdivided into 2 age classes (8–9.86 GW, n = 33 testes from 30 fetuses and 10–12 GW, n = 28 testes from 25 fetuses). ANOVAs with a random embryo effect were performed using unstructured covariance matrices (**, $P < .01$). C, Aspirin dose effect (from 10^{-7} to 10^{-4} M) on testosterone production after 72 hours of exposure (C) by 8 to 9.86 GW (n = 16 testes from 16 fetuses) and 10 to 12 GW (n = 22 testes from 20 fetuses) human fetal testes. Values are expressed as least squares means of testosterone from the respective untreated first day of culture basal sample ± confidence interval (dose effect: $P = .01$ for 8–9.86 GW and $P = .21$ for 10–12 GW). D, After 72 hours of exposure with analgesics, interstitial cells were counted after immunostaining for COUP-TF II. Data are means ± SEM (n = 4–7 testes from 4–7 fetuses of 8–12 GW). A Kruskall-Wallis ANOVA was performed ($P = .14$). NS, not significant.

was (1) to develop an in vitro approach with human fetal testis at different ages and (2) to perform a systematic assessment of the fetal testis endocrine system after exposure to analgesics at concentrations analogous to their therapeutic concentrations or lower (30–32).

Our study reveals that testosterone production by 8 to 12 GW human fetal testis was increased as early as within 48 hours of exposure to indomethacin. The stimulatory effects were still observed after 72 hours with the older testes (10–12 GW), and the amplitude was even higher for the younger testes (8–9 GW). A marked dose-dependent stimulation of testosterone by aspirin was also exclusively seen for the youngest testes (8–9 GW). Of note is the fact that although testosterone production was either unaffected (paracetamol) or stimulated (indomethacin and aspirin) in the fetal testis, it was inhibited by these 3 painkillers in the adult human testis (18), thus demonstrating a major functional difference between human fetal and human adult Leydig cells. The analgesic-induced stimula-

tions of testosterone levels seen for the human fetal testis also contrast markedly with our recent previous in utero and ex vivo fetal rat studies reporting that paracetamol, aspirin, and indomethacin are antiandrogenic and inhibit testosterone production (19, 25). Whether this results from species-specific differences or reflects the differences existing between the rat and human culture systems used remains to be elucidated. Note that such divergences between the effects of diethylstilbestrol and phthalates on the rat (inhibitory) and human fetal testis (no effect) have also recently been shown (27, 33, 36, 37). In contrast, ketoconazole suppressed testosterone production indifferently by the human fetal and adult testis and by the rat fetal testis (18, 19, 25).

This study is the first to investigate the direct production of INSL3 by the fetal human testis, INSL3 being responsible for the outgrowth on the gubernaculum testis. An over time analysis of our data showing that the large interindividual variability of INSL3 production observed
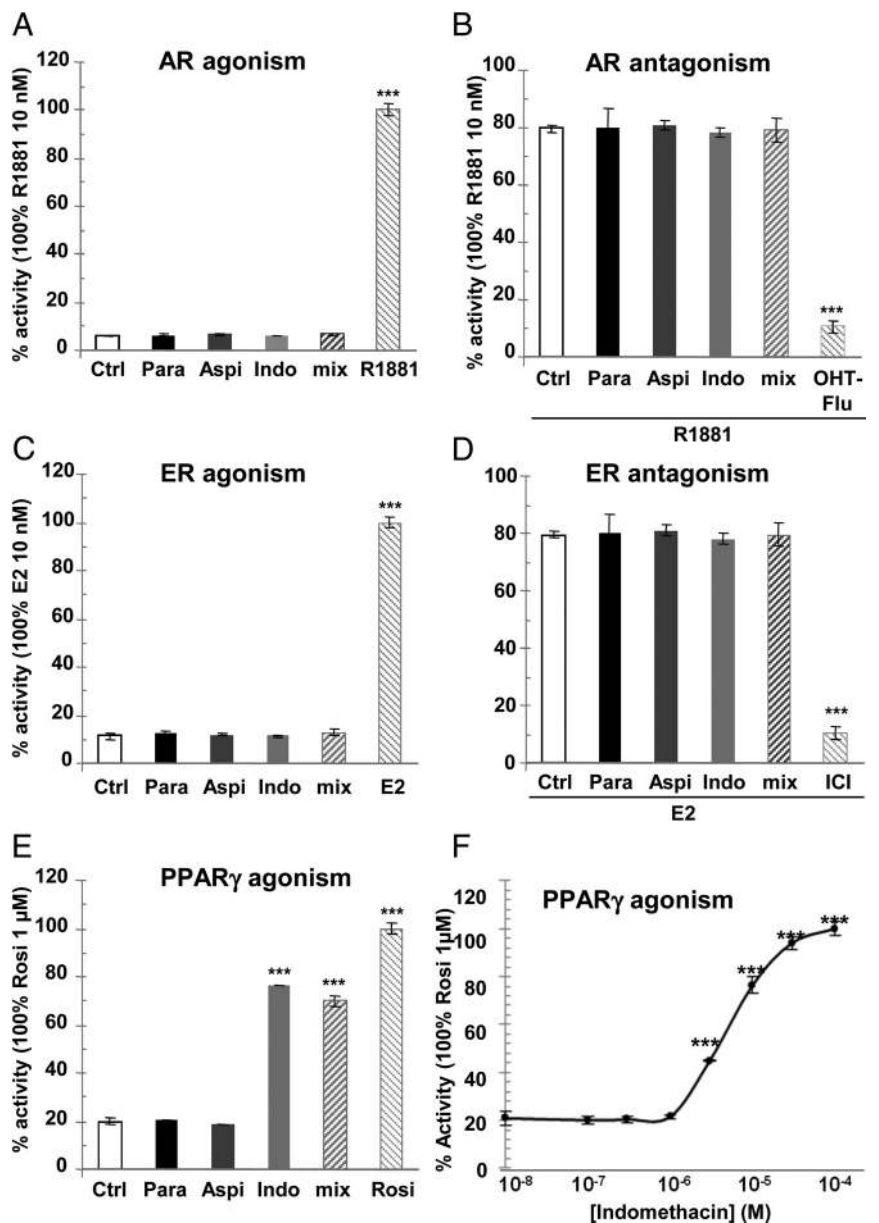
Figure 3. Analgesics and nuclear receptor–mediated activities. The mild analgesics do not display ERα- and AR-mediated activities; indomethacin but not aspirin or paracetamol appears to be a PPARγ agonist. A, HGELN-AR (ERα DNA-binding domain) cells were incubated with DMSO (solvent control [Ctrl]) or 10^{-5} M paracetamol (Para), aspirin (Aspi), indomethacin (Indo), a mixture of these mild analgesics (mix), and the pharmaceutical androgen R1881 (10^{-8} M). B, HGELN-AR (ERα DNA-binding domain) cells were incubated with R1881 at 10^{-9} M in absence or presence of the different analgesics and the pharmaceutical antiandrogen hydroxyflutamide (OUT-Flu, 10^{-5} M). The maximal luciferase activity (100%) was obtained with R1881 at 10^{-8} M C, HGELN-ERα cells were incubated with the different analgesics (10^{-5} M) or estradiol (E2; 10^{-8} M). D, HGELN-ERα cells were incubated with E2 at 10^{-10} M in the absence or presence of the different analgesics and the pharmaceutical antiestrogen ICI172,780 (ICI; 10^{-5} M). The maximal luciferase activity (100%) was obtained with E2 at 10^{-8} M. E, HGELN-GAL4-PPARγ cells were incubated with the different analgesics (10^{-5}M) or the PPARγ agonist rosiglitazone (Rosi; 10^{-6} M). F, Dose-response curve for indomethacin. The maximal luciferase activity (100%) was obtained with rosiglitazone at 10^{-6} M. Values are the means ± SD from 3 separate experiments. ANOVA and the Dunnett multiple comparison test were performed (***, $P < .001$).

blood (38). It is well established that INSL3 is a key factor responsible of the first phase of testicular descent in the mouse (5, 7) and that several mutations of the *INSL3* gene or its receptor were associated with a number of cases of cryptorchidism in humans (15). The period during which the fetal testes were exposed to the analgesics in our experimental model is included in the first phase of testicular descent in human, which occurs between 8 and 17 GW (8, 15). Therefore, the probability is high that the paracetamol-induced dose-dependent inhibition of INSL3 production that we found here is a mechanism by which exposure to paracetamol during pregnancy increases the rate of cryptorchidism in boys (19–21). Aspirin and indomethacin also consistently tended to decrease INSL3 production by first trimester human testes, but the effect was not significant, possibly because of the interindividual variability of the INSL3 levels, as described above.

We demonstrate that the AMH levels nearly doubled after treatment with aspirin, whereas ketoconazole markedly inhibited AMH production. AMH-overexpressing mice display undescended testes and underdeveloped accessory glands, which lead to the consideration that this hormone is a negative regulator of mouse androgen biosynthesis (39). However, our data showing that in the same culture experiments aspirin stimulated both AMH and testosterone levels are in line with Behringer et al (40), suggesting that AMH-induced suppression of androgen biosynthesis cannot account for the inhibition of testicular descent in AMH-overexpressing mice. Patients with genetic defects in the AMH gene or its receptor are affected by cryptorchidism and display abnormal gubernaculum development (41). However, the opponents to the direct implication of AMH in testicular descent argue that by constituting an impassable anatomical

was independent of the age of the fetus led to the conclusion that these variations most likely reflect differences between fetuses, as indicated previously by large variations in the INSL3 levels in the amniotic fluid and cord
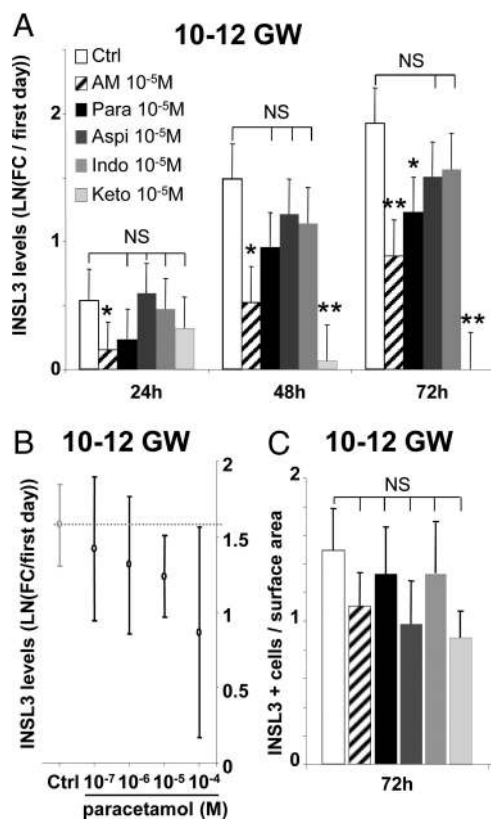
Figure 4. Analgesics and INSL3 production after culture of 10 to 12 GW human fetal testes. A, INSL3 concentrations after culture of 10 to 12 GW human fetal testes in the presence of DMSO (control [Ctrl]) or 10^{-5} M AM404 (AM), paracetamol (Para), aspirin (Aspi), indomethacin (Indo), or ketoconazole (Keto). Results are expressed as the natural logarithm of the fold change from the first day of culture (LN(FC/first day)). Values are least squares means $\pm$ SE of 13 testes from 13 fetuses. Repeated-measures ANOVAs with a random embryo effect were performed using unstructured covariance matrices after natural logarithm transformation. Including an interaction term between time and treatment allowed the estimation of changes in INSL3 levels between treatments and the control within each culture time period (*, $P \leq$ .05 except for the values of paracetamol at 72 h where $P =$ .0543; **, $P <$ .01). B, Paracetamol dose effect (from 10^{-7} to 10^{-4} M) on INSL3 production by 10 to 12 GW human fetal testes. Results are expressed as the natural logarithm of the fold change from the respective first day of culture. Values are least squares means $\pm$ confidence interval of 25 testes from 25 fetuses (dose-effect: $P =$ .02). C, INSL3-positive cell counts after 72 hours of culture in 8.5 to 12 GW human fetal testes treated with DMSO (solvent control) or 10^{-5} M AM404, paracetamol, aspirin, or indomethacin. Data are means $\pm$ SEM (n = 6 testes from 6 fetuses). A Kruskall-Wallis ANOVA was performed ($P =$ .30). NS, not significant.

Figure 5. Analgesics and AMH production after culture of 8 to 12 GW human fetal testes. A, AMH concentrations after culture of 8 to 12 GW human fetal testis in the presence of DMSO (solvent control [Ctrl]) or 10^{-5} M AM404 (AM), paracetamol (Para), aspirin (Aspi), indomethacin (Indo), or ketoconazole (Keto). Results are expressed as fold change from the first day of culture (FC/first day). Values are least squares means $\pm$ SE of 10 to 13 testes from 10 to 13 fetuses. ANOVAs with a random embryo effect were performed using unstructured covariance matrices (**, $P <$ .01); *, $P <$ .05. B, Sertoli cells were identified as AMH-positive cells. Data are means $\pm$ SEM (n = 4–7 testes from 4–7 fetuses). A Kruskall-Wallis ANOVA was performed ($P =$ .18). C, Representative immunostaining of AMH in a 10.29 GW human fetal testis. AMH appears dark (3,3'-diaminobenzidine tetrahydrochloride staining), and sections were counterstained with hematoxylin. Note that the AMH intensity of labeling was consistently and markedly diminished in the ketoconazole-treated explant. Scale bar corresponds to 100 μm.

obstacle to the migration of the gonads, the persistent Müllerian ducts observed both in cases of the so-called persistent Müllerian duct syndrome in humans and in AMH-knockout mice may in itself explain the nondescent of the testes (42).

We demonstrate here that both PGE_2 and PGD_2 are produced by the fetal human testis; this finding therefore opens the way to the study of the role of PGs in this fetal organ in humans and to the identification of possible pathways by which analgesics act on the fetal testis. In contrast
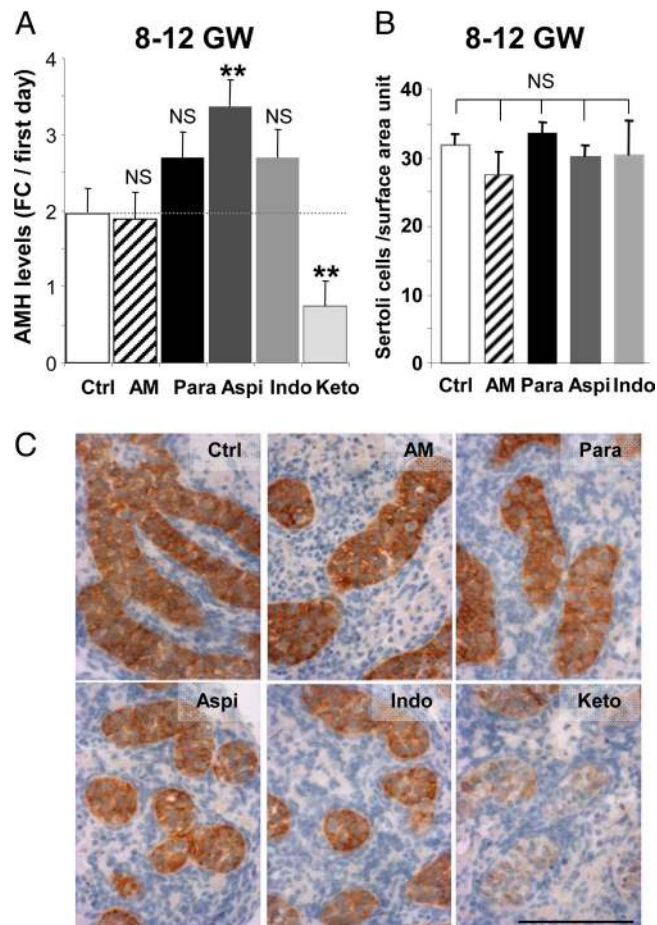
to paracetamol, which inhibited PGE_2 in testes of all ages, the effects of aspirin and indomethacin were only seen in the youngest testes (7–9.86 GW), thus evidencing the critical window of sensitivity for this prostaglandin. It is also noteworthy that in contrast to PGE_2, the human fetal testis production of PGD_2 appeared less sensitive to the effects of analgesics used, the trends for analgesic-induced inhibition consistently observed being not significant. Aspirin and indomethacin are known to act specifically on the cyclooxygenase site of prostaglandin H_2 synthetase (prostaglandin-endoperoxide synthase or prostaglandin G/H
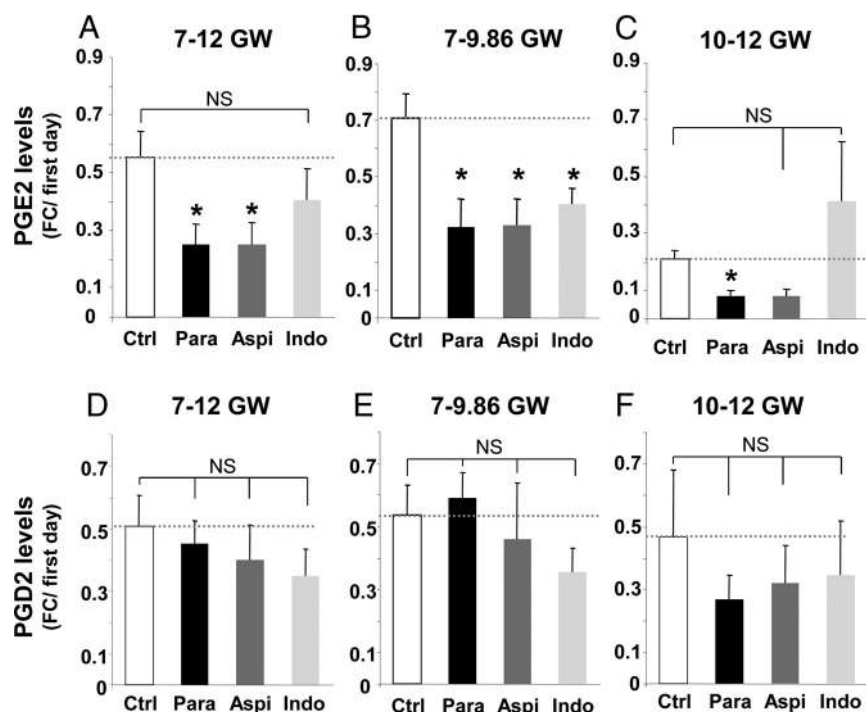
Figure 6. Analgesics and prostaglandin production after culture of 7–12 GW human fetal testes. A–C, PGE$_2$ concentrations after 72 hours of culture of 7 to 12 GW (A), 7 to 9.86 GW (B), and 10 to 12 GW (C) human fetal testis in the presence of DMSO (solvent control [Ctrl]) or 10^{-5} M paracetamol (Para), aspirin (Aspi), or indomethacin (Indo). B and C include age-specific subset of fetuses grouped in A. Results are expressed as fold change from the first day of culture and from control values (FC/first day). Data are means ± SEM (n = 11–13 testes from 11–13 fetuses [A], n = 7–9 testes from 7–9 fetuses [B], and n = 4 testes from 4 fetuses [C]). A Kruskall-Wallis ANOVA was performed followed by a Mann-Whitney rank sum test for comparison with the control (*, P < .05). D–F, PGD$_2$ concentrations after culture of 7 to 12 GW (D), 7 to 9.86 GW (E), and 10 to 12 GW (F) human fetal testis in the presence of the same drugs at 10^{-5} M. E and F include age-specific subset of fetuses grouped in D. Results are expressed as fold change from the first day of culture and from control values. Data are means ± SEM (n = 13–17 testes from 13–17 fetuses [D], n = 7–10 testes from 7–10 fetuses [E], and 6–7 testes from 6–7 fetuses [F]). A Kruskall-Wallis ANOVA was performed (P = .01 and P = .95 for 7–9.86 GW and 10 to 12 GW, respectively) followed by a Mann-Whitney rank sum test for comparison with the control (*, P < .05). NS, not significant.

synthase and cyclooxygenase), whereas paracetamol acts on the peroxidase site of prostaglandin-endoperoxide synthase and thus inhibits the subsequent prostaglandin pathway (43). Of note is the fact that although recent findings indicate that a significant proportion of the mice deficient for the PGD$_2$ synthase gene (*Ptgds*), which encodes for the lipocalin-type PGD$_2$ synthase enzyme responsible for PGD$_2$ synthesis, display unilateral cryptorchidism, thus adding PGD$_2$ as "another piece in the cryptorchidism puzzle" in the mouse (44). These results prompted the same authors to investigate the role of the PGD$_2$ signaling pathway in human testicular descent through the *PTGDS* sequencing of DNA from cryptorchid children; however, they failed to discover any mutation that supported an involvement of this gene in the migration of the testis in humans (44). Although a promising field, the possible relationship that may exist between testicular prostaglan-

dins and testicular descent in humans awaits further investigation.

Work in rodents (in vivo) and with several cell lines from various species suggests that there may be links between PGs and steroids, which differ depending on the species and the cell type considered (9, 45, 46). Exposure of the rat fetal testes and of the adult human testes to paracetamol, aspirin, and indomethacin inhibited production of both testosterone and PGs. However, no clear associations between the patterns of variation of these different hormones were found both in the rat (25, 47) and in humans (18). The same holds true for the relationships between PGs and the other testicular hormones studied, namely INSL3 and AMH, as well as between the latter hormones themselves. Therefore, more work is needed to document what are clearly complex relationships between the different actors of the endocrine system of the human fetal testis.

Investigating several possible pathways underlying the effects of analgesics on the human fetal testis endocrine system in addition to the PG pathways, we found that none of the mild analgesics tested interfered with either the androgen or the estrogen receptors. This therefore excludes the corresponding biological pathways as being involved in the effects of these drugs. However, unlike paracetamol and aspirin, indomethacin appeared to be an effective activator of PPARγ, consistent with previous observations (48), suggesting a possible molecular basis for the particular biological effects of this NSAID in the fetal testis.

Concerning the developmental stage–dependent effects seen on testosterone, and PGE$_2$ levels, the present study is the first direct evidence for the existence of an age window of sensitivity to chemical agents for the responses of the human fetal testis. Such age windows of sensitivity have been suggested previously by epidemiological studies showing that the prevalence of structural anomalies of the testis and of the genital tract (including cryptorchidism) is higher for men exposed to DES before 11 GW than in those who had been exposed later (49). This concept of a

"programming window for reproductive tract masculin-
ization," during which disruption can lead to cryptorchid-
ism has also recently been studied in the rat; extrapolation
of the findings in rat to humans suggests that the window
would be at between 8 and 14 weeks of human gestation
(50). This timing is compatible with both the epidemio-
logical observations mentioned above and with our ex-
periments that have identified slightly different windows
of sensitivity to different treatments, within the 8 to 14
weeks that had been proposed previously as indicated
above (50). Paracetamol, aspirin, and indomethacin have
different endocrine disruptive effects, and these effects de-
pend on the duration of the exposure, the dose, and, for
some hormones, the developmental age. Such a diversity
of effects, some of which are linked to the most crucial
regulatory components of the process of testicular descent,
most probably reflect their generally known diverse mech-
anisms of action (43, 51). This may explain why the com-
bined exposures to different analgesics (eg, aspirin and
paracetamol vs either of them alone) was found to be as-
sociated with a more dramatic risk factor for cryptorchid-
ism compared with the risk factor calculated for each in-
dividual drug (19).

In conclusion, we report that painkillers at doses com-
patible with their therapeutic concentrations have direct
effects on various key aspects of the endocrine function of
the human fetal testis. Our findings may have important
regulatory ramifications. By highlighting the risks to fetal
reproductive health and, in particular, endocrine func-
tions associated with the use of analgesics, our results
should, at minimum, lead to better dissemination of the
existing recommendations for the consumption of anal-
gesics during pregnancy. We also believe that our study
will lead to improvements in these recommendations: the
finding that exposure to mild analgesics during the earliest
fetal stages of development is more likely to be deleterious
to crucial aspects of the fetal testis endocrine system needs
to be taken into account.

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References

1. **Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R.**
Male development of chromosomally female mice transgenic for
Sry. *Nature.* 1991;351:117–121.
2. **Huhtaniemi I.** Fetal testis—a very special endocrine organ. *Eur J
Endocrinol.* 1994;130:25–31.
3. **Jost A, Vigier B, Prépin J, Perchellet JP.** Studies on sex differentiation
in mammals. *Recent Prog Horm Res.* 1973;29:1–41.
4. **Arnold AP.** The organizational-activational hypothesis as the foun-
dation for a unified theory of sexual differentiation of all mamma-
lian tissues. *Horm Behav.* 2009;55:570–578.
5. **Nef S, Parada LF.** Cryptorchidism in mice mutant for Insl3. *Nat
Genet.* 1999;22:295–299.
6. **Spiess AN, Balvers M, Tena-Sempere M, Huhtaniemi I, Parry L, Ivell
R.** Structure and expression of the rat relaxin-like factor (RLF) gene.
Mol Reprod Dev. 1999;54:319–325.
7. **Zimmermann S, Steding G, Emmen JM, et al.** Targeted disruption of
the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol.*
1999;13:681–691.
8. **Bay K, Main KM, Toppari J, Skakkebæk NE.** Testicular descent:
INSL3, testosterone, genes and the intrauterine milieu. *Nat Rev
Urol.* 2011;8:187–196.
9. **Gupta C, Goldman AS.** The arachidonic acid cascade is involved in
the masculinizing action of testosterone on embryonic external gen-
italia in mice. *Proc Natl Acad Sci USA.* 1986;83:4346–4349.
10. **Malki S, Nef S, Notarnicola C, et al.** Prostaglandin D2 induces
nuclear import of the sex-determining factor SOX9 via its cAMP-
PKA phosphorylation. *EMBO J.* 2005;24:1798–1809.
11. **Virtanen HE, Toppari J.** Epidemiology and pathogenesis of cryp-
torchidism. *Hum Reprod Update.* 2008;14:49–58.
12. **Serrano T, Chevrier C, Multigner L, Cordier S, Jégou B.** Interna-
tional geographic correlation study of the prevalence of disorders of
male reproductive health. *Hum Reprod.* 2013;28:1974–1986.
13. **Lee PA, Coughlin MT.** Fertility after bilateral cryptorchidism. Eval-
uation by paternity, hormone, and semen data. *Horm Res.* 2001;
55:28–32.
14. **Prener A, Engholm G, Jensen OM.** Genital anomalies and risk for
testicular cancer in Danish men. *Epidemiology.* 1996;7:14–19.
15. **Foresta C, Zuccarello D, Garolla A, Ferlin A.** Role of hormones,
genes, and environment in human cryptorchidism. *Endocr Rev.*
2008;29:560–580.

16. Toppari J, Larsen JC, Christiansen P, et al. Male reproductive health and environmental xenoestrogens. *Environ Health Perspect*. 1996; 104(suppl 4):741–803.

17. Modick H, Schütze A, Pälmke C, Weiss T, Brüning T, Koch HM. Rapid determination of *N*-acetyl-4-aminophenol (paracetamol) in urine by tandem mass spectrometry coupled with on-line clean-up by two dimensional turbulent flow/reversed phase liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013; 925:33–39.

18. Albert O, Desdoits-Lethimonier C, Lesné L, et al. Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Hum Reprod*. 2013;28:1890–1898.

19. Kristensen DM, Hass U, Lesné L, et al. Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. *Hum Reprod*. 2011;26:235–244.

20. Jensen MS, Rebordosa C, Thulstrup AM, et al. Maternal use of acetaminophen, ibuprofen, and acetylsalicylic acid during pregnancy and risk of cryptorchidism. *Epidemiology*. 2010;21:779–785.

21. Snijder CA, Kortenkamp A, Steegers EA, et al. Intrauterine exposure to mild analgesics during pregnancy and the occurrence of cryptorchidism and hypospadia in the offspring: the Generation R Study. *Hum Reprod*. 2012;27:1191–1201.

22. Berkowitz GS, Lapinski RH. Risk factors for cryptorchidism: a nested case-control study. *Paediatr Perinat Epidemiol*. 1996;10: 39–51.

23. Philippat C, Giorgis-Allemand L, Chevrier C, et al. Analgesics during pregnancy and undescended testis. *Epidemiology*. 2011;22: 747–749.

24. Swan SH, Main KM, Liu F, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113:1056–1061.

25. Kristensen DM, Lesné L, Le Fol V, et al. Paracetamol (acetaminophen), aspirin (acetylsalicylic acid) and indomethacin are anti-androgenic in the rat foetal testis. *Int J Androl*. 2012;35:377–384.

26. Kristensen DM, Skalkam ML, Audouze K, et al. Many putative endocrine disruptors inhibit prostaglandin synthesis. *Environ Health Perspect*. 2011;119:534–541.

27. Hallmark N, Walker M, McKinnell C, et al. Effects of monobutyl and di(*n*-butyl) phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. *Environ Health Perspect*. 2007;115:390–396.

28. Högestätt ED, Jönsson BA, Ermund A, et al. Conversion of acetaminophen to the bioactive *N*-acylethanolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. *J Biol Chem*. 2005;280:31405–31412.

29. Pont A, Williams PL, Azhar S, et al. Ketoconazole blocks testosterone synthesis. *Arch Intern Med*. 1982;142:2137–2140.

30. Rayburn W, Shukla U, Stetson P, Piehl E. Acetaminophen pharmacokinetics: comparison between pregnant and nonpregnant women. *Am J Obstet Gynecol*. 1986;155:1353–1356.

31. Naga Rani MA, Joseph T, Narayanan R. Placental transfer of paracetamol. *J Indian Med Assoc*. 1989;87:182–183.

32. Dalvi SS, Gupta KC, Pohujani SM, Vaidya AB, Satoskar RS. Bioavailability of aspirin after oral and rectal administration in volunteers and patients with fever. *J Postgrad Med*. 1985;31:192–195.

33. Lambrot R, Muczynski V, Lécureuil C, et al. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environ Health Perspect*. 2009;117:32–37.

34. Qin J, Tsai MJ, Tsai SY. Essential roles of COUP-TFII in Leydig cell differentiation and male fertility. *PLoS One*. 2008;3:e3285.

35. Riu A, le Maire A, Grimaldi M, et al. Characterization of novel ligands of ERα, ERβ, and PPARγ: the case of halogenated bisphenol A and their conjugated metabolites. *Toxicol Sci*. 2011;122:372–382.

36. Lassurguère J, Livera G, Habert R, Jégou B. Time- and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. *Toxicol Sci*. 2003;73:160–169.

37. Mitchell RT, Sharpe RM, Anderson RA, et al. Diethylstilboestrol exposure does not reduce testosterone production in human fetal testis xenografts. *PLoS One*. 2013;8:e61726.

38. Anand-Ivell R, Ivell R, Driscoll D, Manson J. Insulin-like factor 3 levels in amniotic fluid of human male fetuses. *Hum Reprod*. 2008; 23:1180–1186.

39. Mendis-Handagama SM, Ariyaratne HB, Fecteau KA, Grizzle JM, Jayasundera NK. Comparison of testis structure, function and thyroid hormone levels in control C57BL/6 mice and anti-Mullerian hormone over expressing mice. *Histol Histopathol*. 2010;25:901–908.

40. Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL. Abnormal sexual development in transgenic mice chronically expressing müllerian inhibiting substance. *Nature*. 1990;345:167–170.

41. Hutson JM, Davidson PM, Reece LA, Baker ML, Zhou BY. Failure of gubernacular development in the persistent müllerian duct syndrome allows herniation of the testes. *Pediatr Surg Int*. 1994;9:544–546.

42. Behringer RR, Finegold MJ, Cate RL. Müllerian-inhibiting substance function during mammalian sexual development. *Cell*. 1994; 79:415–425.

43. Anderson BJ. Paracetamol (acetaminophen): mechanisms of action. *Paediatr Anaesth*. 2008;18:915–921.

44. Philibert P, Boizet-Bonhoure B, Bashamboo A, et al. Unilateral cryptorchidism in mice mutant for Ptgds. *Hum Mutat*. 2013;34:278–282.

45. Andreis PG, Buttazzi P, Tortorella C, et al. The inhibitor of phospholipase-A2, AACOCF3, stimulates steroid secretion by dispersed human and rat adrenocortical cells. *Life Sci*. 1999;64:1287–1294.

46. Dix CJ, Habberfield AD, Sullivan MH, Cooke BA. Inhibition of steroid production in Leydig cells by non-steroidal anti-inflammatory and related compounds: evidence for the involvement of lipoxygenase products in steroidogenesis. *Biochem J*. 1984;219:529–537.

47. Dean A, Mungall W, McKinnell C, Sharpe RM. Prostaglandins, masculinization and its disorders: effects of fetal exposure of the rat to the cyclooxygenase inhibitor- indomethacin. *PLoS One*. 2013; 8:e62556.

48. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem*. 1997;272:3406–3410.

49. Wilcox AJ, Baird DD, Weinberg CR, Hornsby PP, Herbst AL. Fertility in men exposed prenatally to diethylstilbestrol. *N Engl J Med*. 1995;332:1411–1416.

50. Welsh M, Saunders PT, Fisken M, et al. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest*. 2008;118:1479–1490.

51. Pickering G, Moustafa F, Desbrandes S, Cardot JM, Roux D, Dubray C. Paracetamol and opioid pathways: a pilot randomized clinical trial. *Fundam Clin Pharmacol*. 2013;27:339–345.