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 $\gamma$ 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)
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, 20–21). 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/acetosalicylic 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 cultured in each well of 24-well companion plates (Becton-Dickinson). In this case, 4 culture conditions including 1 control were

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designated. 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 μg/mL gentamicin and 2.5 μg/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% CO2. 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 analogues. After 24 hours of culture, the samples were exposed to treatments by adding to the medium, either 5 μL of N-(4-hydroxyphenyl)-arachidonylethanolamide (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 testes (18, 29). Estimating the doses of the analogues 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 μg/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 × 10−5 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, 10−3, and 10−2 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 (MCA22467; 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 (Vectorstain 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 infiltrating 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%; 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 3.0%–13.2% and interassay CV of 5.8–14.2%; AMH/MIS kit; Immunotech). Prostaglandin (PG) D2 and PGE2 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 D2-MOX EIA Kit and Prostaglandin E2 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 H GelN–estradiol receptor α (ERα), H GelN–androgen receptor (AR) (ERα DNA–binding domain) and H GelN-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 β = 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 β = −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).
Analgesics and AMH production

Aspirin strongly stimulated AMH production by Sertoli cells (71%, after a 72-hour exposure; \(P = 0.02\)) (Figure 5A). The consistent trends for AMH production to be increased by paracetamol (40%) and indomethacin (37%) were not significant. None of the analgesics significantly 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 (\(P = 0.114\)) (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...
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 stimulations 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...
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

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Figure 3. Analgesics and nuclear receptor–mediated activities. The mild analgesics do not display ERx- and AR-mediated activities; indomethacin but not aspirin or paracetamol appears to be a PPARγ agonist. A, HGELN-AR (ERx 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 (ERx DNA-binding domain) cells were incubated with R1881 at 10−8 M in absence or presence of the different analgesics and the pharmaceutical antianдроген hydroxyflutamide (OUT-Flu), 10−5 M. The maximal luciferase activity (100%) was obtained with R1881 at 10−8 M. C, HGELN-ERx cells were incubated with the different analgesics (10−5 M) or estradiol (E2, 10−8 M). D, HGELN-ERx cells were incubated with E2 at 10−12 M in the absence or presence of the different analgesics and the pharmaceutical antestrogen ICI172,780 (ICI, 10−5 M). The maximal luciferase activity (100%) was obtained with E2 at 10−8 M. E, HGELN-ERx 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).
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 non-descent of the testes (42).

We demonstrate here that both PGE2 and PGD2 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 PGE2 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 PGE2, the human fetal testis production of PGD2 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 H2 synthetase (prostaglandin-endoperoxide synthase or prostaglandin G/H synthase).
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 PGD2 synthase gene (\textit{Ptgds}), which encodes for the lipocalin-type PGD2 synthase enzyme responsible for PGD2 synthesis, display unilateral cryptorchidism, thus adding PGD2 as “another piece in the cryptorchidism puzzle” in the mouse (44). These results prompted the same authors to investigate the role of the PGD2 signaling pathway in human testicular descent through the \textit{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 prostaglandins 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 PPAR\~\gamma, 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 masculinization,” during which disruption can lead to cryptorchidism 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 epidemiological observations mentioned above and with our experiments 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 depend 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 mechanisms of action (43, 51). This may explain why the combined exposures to different analgesics (eg, aspirin and paracetamol vs either of them alone) was found to be associated with a more dramatic risk factor for cryptorchidism compared with the risk factor calculated for each individual drug (19).

In conclusion, we report that painkillers at doses compatible 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 functions associated with the use of analgesics, our results should, at minimum, lead to better dissemination of the existing recommendations for the consumption of analgesics 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.

Acknowledgments

We thank all the staff of the Department of Obstetrics and Gynecology and the Department of Pediatric Surgery of the Rennes Sud Hospital (Rennes, France) and the participating women, without whom this study would not have been possible. We thank Marina Grimaldi of the Institut de Recherches Cliniques de Montréal–Institut National de la Santé et de la Recherche Médicale U896 for her contribution to the experiments with nuclear receptor-mediated activities.

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This study was funded by Institut National de la Santé et de la Recherche Médicale, University of Rennes 1, École des Hautes Études en Santé Publique—School of Public Health, by grants from the Agence Nationale de Sécurité du Médicament et des Produits de Santé (AAP-2012-037), and the Danish Council for Independent Research (Medical Sciences). No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

B.J. and S.M.-G. designed the research. S.M.-G., C.N.N., C.D.-L., I.C., and M.B.M. performed the research. P.B. designed the nuclear receptor–mediated activity experiments. P.P. and V.L. supervised the collection of the first trimester human fetal testis samples. M.B.M. and B.J. supervised the collection of the human gubernaculum testis samples. C.C. supervised and performed statistical analysis. N.D.-R. and D.M.K. contributed to critical discussions. All authors contributed to critically reviewing the draft manuscript, which was elaborated mainly by B.J. All authors saw and approved the final version of the report.

Disclosure Summary: The authors have nothing to disclose.

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