Prevalence of hypospadias in grandsons of women exposed to diethylstilbestrol during pregnancy: a multigenerational national cohort study

Prenatal diethylstilbestrol (DES)-exposed mice have raised the suspicion of a transgenerational effect in the occurrence of genital malformation in males. This nationwide cohort study in collaboration with a French association of DES-exposed women studied 529 families and showed that a significant proportion of boys born to DES daughters exhibited hypospadias with no other molecular defects identified. (Fertil Steril® 2011;■:■–■. ©2011 by American Society for Reproductive Medicine.)

Key Words: Hypospadias, DES, diethylstilbestrol, estrogens, prevalence, environment, epigenetic

Although the role of fetal androgens is crucial to male genital development during the first trimester of pregnancy, defects in the synthesis or molecular action of testosterone are rare in isolated hypospadias (1). Hypospadias may be a multifactorial defect arising from genetic, hormonal, and environmental factors (2–4). It has been hypothesized that changes in androgen/estrogen balance due to endogenous or exogenous hormonal factors during the critical period of penile and urethral development contribute to this malformation (5, 6).

Men who were exposed in utero to diethylstilbestrol (DES), a synthetic estrogen, may exemplify the effects of environmental chemicals with endocrine-disrupting activity on genital development (7). DES was prescribed for pregnant women from the late 1930s to the 1970s in the mistaken belief that it would prevent miscarriage or premature birth. Unfortunately, DES was found to be not only ineffective but also harmful. Daughters born from DES-related pregnancies often show abnormalities in the Müllerian structures and have elevated risks of peripubertal vaginal and cervical clear-cell adenocarcinoma, fertility problems, ectopic pregnancies, miscarriages, and premature births (8). The risk of reproductive tract abnormalities also appears to be increased for DES sons, who may present hypoplastic testis, epididymal cysts, cryptorchidism, or hypospadias (9).

After several studies in animals (10–12), a question emerged as to whether the harmful effects of DES can be "transmitted" to subsequent generations. Newbold and colleagues (10, 11) reported an increased incidence of reproductive tract tumors in male and female descendants of mice developmentally exposed to DES. In the human, Klip et al. (13) reported an increased risk of hypospadias in sons of DES daughters in a cohort of women diagnosed with fertility problems. Other studies either confirmed (14, 15) or questioned (16) these results. However, the direct implication of DES in the occurrence of hypospadias remains debatable, since many other uncontrolled factors, especially environmental and genetic, are implicated in this malformation (3). We studied the prevalence of hypospadias in the grandsons of DES-treated and -untreated women and ruled out other environmental and genetic factors that could have been associated with the malformation in these patients.

A nationwide cohort study was conducted in collaboration with a French association of DES-treated women (HHORAGES Association). The reason the women joined this association was *not* the presence of hypospadias in the second or third generation but rather psychological disturbances, vaginal and cervical clear-cell

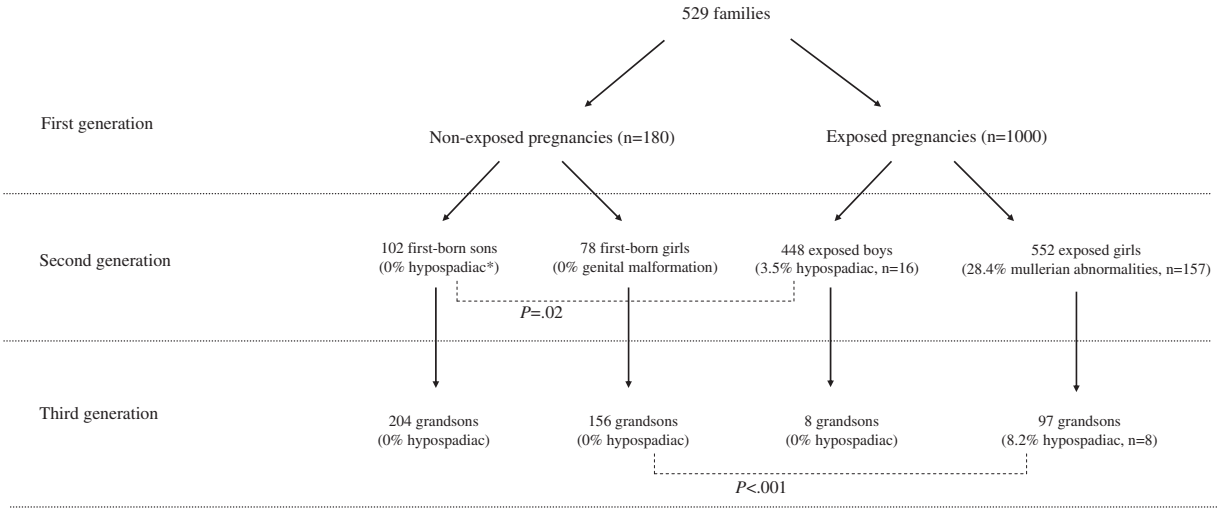
Nicolas Kalfa, M.D., Ph.D.[a,b,c]
Françoise Paris, M.D.[a,b]
Marie-Odile Soyer-Gobillard, Ph.D.[d]
Jean-Pierre Daures, M.D., Ph.D.[e]
Charles Sultan, M.D., Ph.D.[a,b]

[a] *Unité d'Endocrinologie-Gynécologie Pédiatrique, Service de Pédiatrie I, Hôpital Arnaud de Villeneuve, CHU Montpellier and Université Montpellier, France*
[b] *Service d'Hormonologie (Développement et Reproduction), Hôpital Lapeyronie, CHU Montpellier and Université Montpellier, France*
[c] *Service de Chirurgie et Urologie Pédiatrique, Hôpital Lapeyronie, CHU Montpellier and Université Montpellier, France*
[d] *Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7628, Université Paris VI et Association Hhorages-France, Drancy, France*
[e] *Département de Biostatistiques, Institut de Recherche Clinique, Université de Montpellier, France*

Received October 14, 2010; revised February 21, 2011; accepted February 23, 2011.
N.K. has nothing to disclose. F.P. has nothing to disclose. M.-O.S.-G. has nothing to disclose. J.-P.D. has nothing to disclose. C.S. has nothing to disclose.
The first two authors contributed equally to this article.
This study was supported by University grant no. PHRC UF8270.
Reprint requests: Charles Sultan, M.D., Ph.D., Unité d'Endocrinologie-Gynécologie Pédiatriques, Service de Pédiatrie 1, Hôpital Arnaud de Villeneuve, CHU Montpellier, 34295 Montpellier, France (E-mail: c-sultan@chu-montpellier.fr).

FIGURE 1

Detailed patient groups included in the study. In the second generation, the phenotype of affected boys was isolated hypospadias in all cases, severe in 12 cases (posterior or penoscrotal), and not severe in four cases (mild or anterior). In the third generation, the hypospadias was severe in five cases and not severe in three cases. Bilateral cryptorchidism was present in one case. *The size of the population was under 1,000, and the prevalence of hypospadias of about 1/1,000, as seen in the general population, could not be represented.



Kalfa. DES and third-generation hypospadias. Fertil Steril 2011.

adenocarcinomas, miscarriages, and other abnormalities. Five hundred twenty-nine families were included. All of the second- and third-generation offspring were accounted for and included in the study. No one declined to participate. DES exposure was reported in 1,000 out of 1,180 pregnancies. Figure 1 details the patient groups. The clinical diagnosis of hypospadias was standardized and based on a detailed operative report or direct clinical examination by a pediatrician and/or urologist. The malformation was characterized as severe (proximal, penoscrotal) or nonsevere (glandular, subcoronal, distal, midshaft). Each mother with a hypospadiac son was contacted and responded to a short questionnaire validated in Europe for data collection (no. QLK4-1999-01422) to determine whether other occupational exposure had occurred during the pregnancy. To exclude a defect of the androgen pathway, we performed molecular analysis of the genes known to be associated with hypospadias such as androgen receptor (AR), 5α reductase (srd5A2), and MAMLD1 genes in DNA from peripheral blood, as described elsewhere (17–19). The local university hospital ethics committee approved this study (ID RCB No. 2008-A00781-54), and each patient gave informed consent through the Hhorage Association.

The prevalence of hypospadias was low in boys unexposed to DES in utero (0/180), whereas it was high in the in utero–exposed boys (3.57%, 16/448, P=.02). In the third generation, the prevalence of hypospadias in boys born to DES daughters was high when compared with boys born to unexposed parents (8.2%, n = 8/97 vs. n = 0/360; P<.001). The hypospadiac patients of the second and third generations were not related. The results are summarized in Figure 1.

Neither mutations nor polymorphisms of the AR and MAMLD1 genes were found among hypospadiac boys of the third generation. Only one polymorphism of the srd5A2 gene was detected (A49T) in a boy. The mothers of the third-generation affected boys indicated little environmental or occupational exposure to endocrine-disrupting chemicals during pregnancy (no professional activity, n = 2; sales clerk in a food or clothing shop, n = 3; office worker, n = 3), and such exposure was therefore unlikely to have contributed to the occurrence of hypospadias. Two mothers of hypospadiac sons exhibited hypoplastic or bifid uterus.

The main effect of DES is profound disturbance in the androgenic/estrogenic balance of animal and human fetuses since it has both estrogenic and antiandrogenic actions by competing with natural androgens for the ligand-binding domain of the androgen receptor (20, 21). In utero exposure to DES during the critical period of reproductive tract development is known to induce genital malformation in mice (11, 22). In utero–exposed sons show greater risks of structural urogenital abnormalities like hypospadias, epididymal cysts, micropenis, and cryptorchidism (23). The present study reinforces these data with a prevalence of hypospadias greater than 3%, although it should be noted that the second-generation population included only 180 controls from the same families since this series was specifically designed to study the third-generation boys.

More interesting is the hypothesis of a transgenerational effect of DES. Animal studies first suggested that DES might increase transgenerational susceptibility to malignant tumors of the female reproductive tract, presumably by damage to germ cells and abnormal imprinting (11). In human beings, DES exposure may also lead to permanently altered germ cells (24). The suggestion of a transgenerational effect of DES in human beings was based on the observation of a high prevalence of hypospadias, particularly with severe phenotypes, in the sons of women exposed to DES in utero (13).

But variations in the definition of the control population may explain the wide range of odds ratios reported in the literature to date. Palmer et al. (16) reported a prevalence 6 times higher than that of Klip et al. (13). The present study, which shows a high prevalence of hypospadias of various severities in the third generation, tried to limit this bias and included DES-free pregnancies and DES-exposed pregnancies from the same families. Nevertheless, two limitations should be noted: (1) DES-exposed women without problems were not included; and (2) the fertility status of the exposed and nonexposed couples, the age at pregnancy, and the parity for each women were variable, and this may have hidden fertility problems or greater use of contraception. The low fertility rates of the DES sons may also be explained by other findings, such as severe psychotic disorders (25) or oligospermia in cases of hypospadias with additional defects (26).

We did not identify any genetic or environmental factors that would have explained the hypospadias in DES grandsons. Our results thus raise the question of the mechanism through which DES causes adverse effects in subsequent generations. The frequency of transmission both observed in our series of hypospadiac grandsons and previously reported in generations examined for various disease states secondary to DES exposure is particularly high. This frequency of a transgenerational phenotype is such that a mutational event involving the DNA sequence could not be implicated (27, 28). DES-induced changes in epigenetic background and alteration of DNA methylation could be significant factors in the susceptibility to disease development. The primordial germ cells undergo demethylation during migration and early colonization of the embryonic gonad, followed by remethylation starting at the time of sex determination in a sex-specific manner (29–31). The pregnant mother's exposure to DES at the time of fetal sex determination appears to be sufficient to alter the remethylation of the germ line in the male fetus and permanently reprogram the imprinted pattern of DNA methylation (32). The transmission of multigenerational DES effects would thus occur through the paternal lineage (12, 33). But our findings indicated that most of the third-generation hypospadiac boys were born to DES daughters. This agreed with previous studies (14), although paternal transmission of DES effects is not excluded (15). Epigenetic changes in the AR gene, transmitted through the DES daughter, could explain such a finding since the antiandrogen effect of DES is known to modify the phosphorylation level of AR (34).

The association between hypospadias in grandsons and uterine abnormalities in their mothers suggests other hypotheses for the transgenerational mechanisms of DES. First, DES daughters may have displayed disturbed hormonal balance during their reproductive life or placental malfunction that might have interfered with the genital development of a male fetus. Second, the estrogen receptor gene ERα and estrogen-responsive genes that contribute to the development of both female internal genitalia and hypospadias (35, 36) may also be involved since ERα is implicated in the induction of abnormalities after DES exposure (37, 38). Last, the genes involved in the structural differentiation of both the female and male reproductive tracts may be altered by DES exposure. DES has been reported to delay expression of HOXa family genes during Müllerian duct development (39). DES could also interfere with HOX gene expression during penile formation (40).

For many authors, DES is an experimental environmental xenoestrogen (41). Despite the bias that could not be fully eliminated and the difficulty of extrapolating the risks of exposure (no monotonic dose-response relationship, varying effects depending on the timing of exposure in the developing organism, manifestations delayed until later in life), this clinical study strengthens the suspicion of the transgenerational effects of environmental endocrine disruptors.

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