Effect of in-utero diethylstilboestrol exposure on human oocyte quality and fertilization in a programme of in-vitro fertilization

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Genital tract abnormalities and adverse pregnancy outcome are well known in women exposed *in utero* to diethylstilboestrol (DES). Data about adverse reproductive performance in women exposed to DES have been published, including controversial reports of menstrual dysfunction, poor responses after ovarian stimulation, oocyte maturation and fertilization abnormalities. We compared oocyte quality, in-vitro fertilization results and embryo quality for women exposed *in utero* to DES with a control group. Between 1989 and 1996, 56 DES-exposed women who had 125 in-vitro fertilization (IVF) attempts were retrospectively compared to a control group of 45 women with tubal disease, who underwent 73 IVF attempts. Couples suffering from male infertility were excluded. The parameters compared were oocyte quality (maturation abnormalities, immature oocyte, mature oocyte), fertilization and cleavage rate (per treated and metaphase II oocytes), and embryo quality (number and grade). We found no significant difference in oocyte maturational status, fertilization rates, cleavage rates, embryo quality and development between DES-exposed subjects and control subjects. These results suggest that in-utero exposure to DES has no significant influence on oocyte quality and fertilization ability as judged during IVF attempts.
Key words: diethylstilboestrol/embryo/infertility/in-vitro fertilization/oocytes

Introduction

Women exposed *in utero* to diethylstilboestrol (DES) exhibit genital tract abnormalities including vaginal adenosis, cervical ectropion, ridges, pseudopolyps, uterine hypoplasia and in some cases, vaginal and cervical clear cell adenocarcinoma. These women may suffer pregnancy complications as a result of reproductive tract abnormalities such as uterine defects, including a T-shaped or hypoplastic cavity, a septate uterus, intrauterine synechiae or irregular uterine margins (Kaufman *et al.*, 1977; Siegler *et al.*, 1979; Cabau *et al.*, 1984; Epelboin and Bulwa, 1993; Noyes *et al.*, 1996; Salle *et al.*, 1996). These are associated with an increased incidence of poor reproductive outcome (Senekjian *et al.*, 1988). Among women pregnant

for the first time, adverse pregnancy outcomes, including spontaneous abortion (Sandberg *et al.*, 1980; Cabau, 1989), ectopic pregnancy (Kaufman *et al.*, 1986) and perinatal death, were more frequent in DES-exposed women than unexposed women (Herbst *et al.*, 1980). Data concerning adverse reproductive performance have been published and include controversial reports of oocyte maturation abnormalities in mice exposed to DES (MacLachlan *et al.*, 1980; Iguchi *et al.*, 1990, 1991) or menstrual dysfunction (Bibbo *et al.*, 1977; Barnes, 1979) and increased occurrence of endometriosis (Stillman and Miller, 1984; Berger and Alper, 1986; Senekjian *et al.*, 1988) or autoimmune disease (Way *et al.*, 1987; Noller *et al.*, 1988) in women exposed to DES. It is not known if in-utero exposure to DES leads to abnormalities of the oocyte in adulthood.

The aim of this study was to investigate oocyte quality, fertilization and embryo quality in women exposed to DES by comparing such women participating in an in-vitro fertilization (IVF) programme to a control group.

Materials and methods

Patients

All women with a history of in-utero diethylstilboestrol exposure who participated in an IVF programme between 1989 and 1996, were retrospectively included in the study group. The women of the control group were selected as follows: for each woman in the study group, the first woman of the same age and with tubal infertility who had an IVF attempt within the 3 subsequent months was included. In the DES group, the women had many genital abnormalities in their medical history (Table I). The incidence of ovulatory dysfunction (defined as any history of irregular menstrual cycle with luteal phase defects) and endometriosis was much higher in the study group. However, since no differences were found in the oocyte number and quality and pregnancy rate in women with or without these defects in the DES-exposed group, it was decided not to take these factors into account when matching the subjects included in the control group. If there was a suspicion of male infertility, based on abnormal semen parameters according to World Health Organization (WHO) criteria (1992), the couple was excluded to avoid bias due to the male factor.

There were 125 IVF attempts with oocytes collected from 56 DES-exposed women. The control group included 45 women and 73 IVF attempts. The whole population and two more homogeneous subgroups were analysed. The first one considered only the first IVF attempt for each patient, the second included only patients ≤35 years old for whom IVF was attempted between January 1995 and June 1996 (Table II).

Ovarian stimulation and oocyte recovery

The protocols for ovarian stimulation involved associating long or short gonadotrophin-releasing hormone agonist (GnRHa) treatment

Table I. Reproductive history and genital abnormalities of the 56 women exposed to diethylstilboestrol (DES) and the 45 women with tubal occlusion in the control group

	DES		Control group	
	n	%	*n*	%
Primary infertility	27	48	14	31
Secondary infertility	29	52	31	69
Ovulatory dysfunction	32	57	–	–
History of ovarian cysts	8	14	2	4
Endometriosis	9	16	3	7
Cervical mucus defect	22	39	–	–
Cervical stenosis	31	55	–	–
Uterine abnormalities	34	61	–	–
Bilateral hydrosalpinges	–	–	8	18
Bilateral tubal occlusion	15	27	45	100

Figure 1. Mean fertilization rates and cleavage rates after IVF in diethylstilboestrol (DES)-exposed women (■) or controls (□).

and administration of gonadotrophins. For the long protocol, the women were down-regulated with GnRHa (triptoreline or leuproreline) and thereafter stimulated with daily injections of human menopausal gonadotrophin (HMG) or purified follicle stimulating hormone (FSH). For the short protocol, GnRHa was started on day 1 of menstruation and HMG was added on day 3 (Frydman *et al.*, 1988). The short protocol was used for 5% (*n* = 6) of the DES-exposed group and 14% (*n* = 12) of the control group. Ovulation was induced with 5000 IU of human chorionic gonadotrophin (HCG). Oocytes were harvested 35–37 h after HCG injection using a transvaginal ultrasound procedure (Frydman *et al.*, 1988).
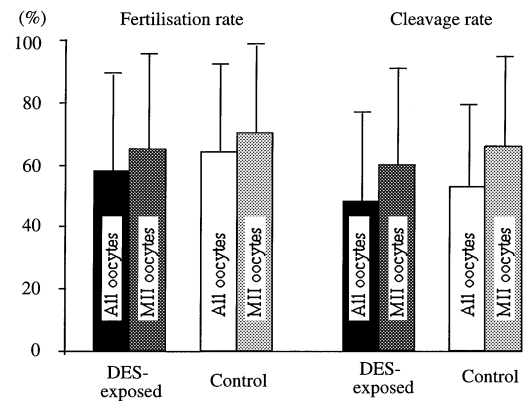
All the oocyte–cumulus complexes except those with 'fractured zona pellucida' and degenerating oocytes were washed and incubated in B2 medium (CCD, Paris, France) before insemination.

Sperm preparation

Patients' semen was collected by masturbation on the day of IVF and allowed to liquefy for 30 min at 37°C. The sperm samples were prepared by a conventional swim-up procedure in 31% of cases and by centrifugation through a mini-Percoll gradient in 69% of cases (Ng *et al.*, 1992).

IVF procedure

From 1989 to 1994, oocytes were classified as mature if they were fully enclosed by expanded radiant corona and cumulus cells (type I), or as immature if the cumulus was partly expanded (type II) or non-expanded with a compact layer of corona cells (type III). Four oocytes per well were inseminated with 30 000–60 000 spermatozoa in 1 ml of B2 medium using a multidish four-well system. After 1994, the cumuli were placed into 30 µl drops of B2 medium under equilibrated mineral oil (Merck, Nogent sur Marne, France) and inseminated 3 h after the sperm preparation with 5000 motile spermatozoa. All oocytes were incubated and inseminated in separate microdrops to allow for individual examination and follow-up. Gametes were cultured in a humidified gas incubator at 37°C (5% CO_2, 95% air). At ~17–20 h post-insemination, the corona cells were removed mechanically by repeated gentle aspiration and expulsion of the oocyte through a Pasteur pipette. The zygotes were transferred into sperm-free B2 medium and observed to determine the pronuclear status of the zygote and the nuclear status of non-fertilized oocytes using a Nikon Diaphot inverted microscope with phase contrast optics, at ×400 magnification. Abnormal oocytes with a fractured zona pellucida, atretic oocytes and immature oocytes at the germinal vesicle stage or at metaphase I were recorded. Unfertil-

ized mature oocytes at metaphase II were recognized by the first polar body and the absence of pronuclei.

Forty-eight hours after insemination, cleaved embryos were graded according to their morphological appearance using the following scale: type A (no extracellular cytoplasmic fragments), type B (embryo fragmentation of 1–20%), type C (embryo fragmentation rate 21–50%), and type D (embryo fragmentation rate >50%).

Statistical analysis

Student's *t*-test was used for statistical evaluation. Statistical significance was defined as a *P* value < 0.05.

Results

Oocytes from the whole DES-exposed population corresponding to 125 IVF attempts were compared with those from the control group corresponding to 73 IVF attempts, as shown in Table III. The mean number of oocytes collected from DES-exposed women was slightly but not significantly lower than that from unexposed women. For the DES-exposed group, the percentages of mature type I (79.5%) and immature type II (20.5%) cumulus and corona cells were not significantly different from those for the control group (75.6 and 24.4% respectively). The mean number of abnormal or immature oocytes was also not different between the groups. This was also true when subgroups of younger women or only the first IVF attempt only were considered.

The fertilization rates measured 18–24 h after insemination were slightly lower, but not significantly, for DES-exposed women than unexposed women (Figure 1). The percentage of zygotes with three pronuclei per mature oocyte for DES-exposed women (2.3%) was also not different from that for unexposed women (2.1%). DES exposure did not affect the cleavage rate. Although the proportion of embryos with >50% fragmentation was higher for the DES-exposed group, there was no significant difference in the proportions of fragmented embryos 2 days after insemination between DES-exposed and control groups (Figure 2). Embryo quality and development as judged by the percentage of embryos which reached the type A or B four-cell stage were not significantly different in DES-exposed subjects (22.2%) and in control subjects (16.3%). An embryo transfer occurred in 91.2% of IVF cycles in DES-exposed women and 87.7% of the cycles in the control group.

Table II. Characteristics of the whole population exposed to diethylstilboestrol (DES) and control groups, and also for subgroups of women having their first IVF attempt and those aged ≤35 years

| | All subjects | | Subgroups | | | |
| | | | First IVF | | ≤35 years, 1995–1996 | |
	DES-exposed	Control	DES-exposed	Control	DES-exposed	Control
Number of patients	56	45	54	28	17	25
Age (years)	35.2 ± 4.2*	34.5 ± 4.5	36.1 ± 4.59	34.6 ± 7.88	30.8 ± 1.68	32.2 ± 6.15
	(27–44[†])	(26–42)	(27–44)	(28–42)	(27–34)	(26–35)
Number of IVF attempts	125	73	54	28	23	30

*Mean ± SD.
[†]Range.

Table III. Comparison of oocyte recovery and quality in women exposed to diethylstilboestrol (DES) and controls

| | All subjects | | Subgroups | | | |
| | | | First IVF | | ≤35 years, 1995–1996 | |
Oocyte quality	DES-exposed	Control	DES-exposed	Control	DES-exposed	Control
Retrieved oocytes	9.1 ± 8.36*	10.8 ± 9.06	8.36 ± 7.65	10.04 ± 8.61	9.46 ± 6.45	12.53 ± 7.83
Atretic oocytes	0.21 ± 0.7	0.59 ± 1.08	0.33 ± 0.9	0.33 ± 0.76	0.7 ± 1.21	1.1 ± 1.42
Fractured zona pellucida	0.69 ± 1.44	0.91 ± 1.4	0.7 ± 1.23	0.75 ± 1.48	0.5 ± 0.11	1.23 ± 1.52
Germinal vesicle stage	0.9 ± 1.1	0.33 ± 0.57	0	0	0.84 ± 1.06	0
Metaphase I	0.26 ± 0.65	0.71 ± 0.99	0.29 ± 0.7	0.46 ± 0.74	0.34 ± 0.71	1.1 ± 1.09
Metaphase II	7.04 ± 4.47	8.26 ± 5.02	7.04 ± 4.82	8.5 ± 5.63	7.08 ± 3.36	9.1 ± 3.8

*Mean ± SD.
There were no significant differences between the DES-exposed women and controls.
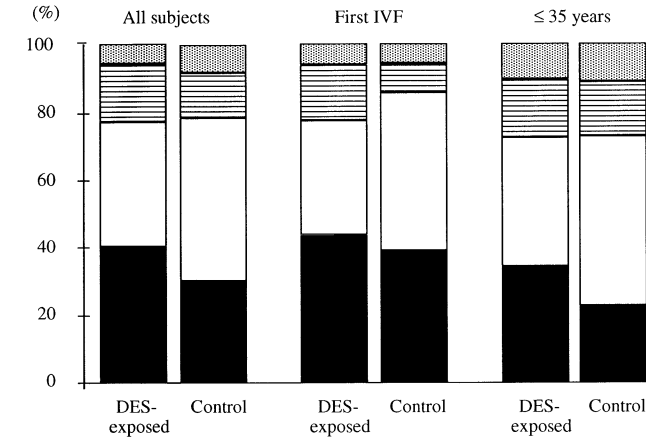
Figure 2. Embryo quality 2 days after insemination in diethylstilboestrol (DES)-exposed women and controls (■ type A: 0% fragmentation, □ type B = 1–20%, ▤ type C = 21–50%, and ▨ type D ≥51%).

Although not significantly different, the clinical pregnancy rate was lower in the DES-exposed group (15.8%) compared to the control group (23.1%), but the mean number of replaced embryos was also lower (2.5 ± 1.11 versus 2.9 ± 1.11).

Discussion

A similar incidence of oocyte anomalies, IVF and cleavage rates, and clinical pregnancy rates was found for women exposed to DES *in utero* and a control group of unexposed women.

In mice, it has been demonstrated (MacLachlan *et al.*, 1980) that an abnormally low number of oocytes are recovered and a large number of degenerating oocytes found after ovarian stimulation in females previously exposed to DES *in utero*. It has also been found (Iguchi *et al.*, 1991) that mouse oocyte maturation is affected by perinatally administered DES. Neonatal exposure leads to larger gap junctions in the granulosa cells of mature follicles and a stronger attachment among granulosa cells, which prevents disaggregation of the cumulus–oophorus complex, even after ovulatory stimuli. DES-exposed mice also discharged a similar number of ova to control mice following stimulation by gonadotrophins, and oocytes from polyovular follicles in DES-exposed mice had a significantly decreased fertilization capacity *in vitro*.

Data concerning the effects of DES on women's fertility are conflicting. According to previous studies (Senekjian *et al.*, 1988), primary infertility was significantly more frequent among women who had been exposed to DES than among unexposed women. Other studies (Barnes *et al.*, 1980; Cousins *et al.*, 1980; Stillman, 1982) did not find differences in fertility rates between DES-exposed women and unexposed subjects, as estimated by the number of pregnancies per woman. It has been suggested (Bibbo *et al.*, 1977) that DES exposure may be associated with menstrual irregularities, but other studies (Barnes, 1979) revealed no significant irregularities at either initial or follow-up examination. During IVF procedures some studies (Muasher *et al.*, 1984; Karande *et al.*, 1990) did not find significant differences in preovulatory, immature and degenerated oocytes between patients with tubal disease and

DES-exposed women. However, other studies (Sangvai *et al*., 1996) found a similar incidence of diminished ovarian reserve and similar follicular recruitment with gonadotrophins in DES-exposed and other women. In the group of in-utero DES-exposed women analysed here, the prevalence of ovulatory dysfunction and endometriosis was very high, but the number and quality of oocytes retrieved after stimulation were similar to those from women not exposed to DES. This suggests that the exposure to DES *in utero* did not alter the development of the oocytes in the ovary and that those oocytes are not refractory to the maturation stimulus and/or to the removal of inhibitory substances that maintain the oocyte in the germinal vesicle stage. The fertilization ability and the embryo formation also indicate a good qualitative maturation of the oocytes retrieved, but factors such as aneuploidy, embryonic genome expression and ultrastructure have not been assessed in this study.

The clinical pregnancy rate (number of cycles with fetal sacs on ultrasound) per embryo transfer was not significantly different between the two groups although lower in the IVF cycles performed in DES-exposed patients. Thus, lower delivery rate could be related to other important variables such as uterine defects and endometrial morphology and thickness as previously reported (Kaufman *et al*., 1977; Siegler *et al*., 1979; Cabau, 1984; Epelboin and Bulwa, 1993; Noyes *et al*., 1996; Salle *et al*., 1996).

Acknowledgements

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