Superovulation and early embryo development in the adult mouse after prenatal exposure to diethylstilboestrol

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Summary. Pregnant mice were injected subcutaneously with diethylstilboestrol (DES: 10 µg/kg body weight in 0.1 ml corn oil) or corn oil alone on Day 15 or 16 of gestation (Day 1 = day of copulatory plug) and allowed to give birth. Female progeny from control and DES-exposed animals were superovulated with exogenous gonadotrophins at 6–8 weeks of age. In-vivo results indicated that the total number of ovulated ova, 2-cell embryos and blastocysts were significantly increased in DES-exposed progeny but that there was a decline in developmental potential from the ovulated ova stage to the blastocyst stage in these animals. However, there was no significant difference in the in-vitro development of 2-cell embryos to the blastocyst stage between control and DES-exposed animals. These results indicate that the ovaries of mice exposed *in utero* to DES are capable of responding to exogenous gonadotrophins and that second generation progeny have the potential for normal development to the early post-blastocyst stage of embryogenesis. The in-vivo decline in developmental potential may be attributable to reproductive tract abnormalities rather than ova/embryo defects.

Keywords: diethylstilboestrol; mouse; embryo; *in vitro*; development

Introduction

In humans, exposure to diethylstilboestrol during gestation results in reproductive tract abnormalities, including the rare occurrence of adenocarcinoma of the vagina (Herbst *et al.*, 1971). The mouse is suitable for examination of specific short- and long-term effects of prenatal or neonatal exposure to diethylstilboestrol (Bern *et al.*, 1976; McLachlan, 1977; McLachlan *et al.*, 1980; Ennis & Davies, 1982). Abnormalities in the mouse vagina (Plapinger & Bern, 1979), cervix (Bern *et al.*, 1976), oviduct and endometrium (Wordinger & Morrill, 1985) have been reported after prenatal or neonatal exposure to diethylstilboestrol. Some of these changes may be ovarian dependent since female progeny of diethylstilboestrol-treated mice are often in a persistent oestrogenic state even at 12–18 months of age (McLachlan *et al.*, 1980). The ovarian morphology of mice exposed to diethylstilboestrol *in utero* is consistent with this suggestion in that ovaries contained numerous follicles in various stages of growth and atresia but lacked corpora lutea (Wordinger & Highman, 1984).

Insufficient information is available concerning the functionality of the ovaries and the developmental potential of ova (i.e. second generation progeny) from animals exposed to diethylstilboestrol. It is also not apparent whether the reproductive tract of such progeny would be favourable for early embryonic development and ova transport. Answers to these questions are important since increased infertility (Herbst *et al.*, 1980; Stillman, 1982), spontaneous abortions (Herbst *et al.*, 1980; Barnes *et al.*, 1980) and ectopic pregnancies (Cousins *et al.*, 1980; Berger & Goldstein, 1980) have been reported in women exposed to diethylstilboestrol during gestation.

The objectives of this study were to (1) ascertain whether the ovaries from progeny of mice exposed to diethylstilboestrol could respond to exogenous gonadotrophins, (2) establish whether ovulated ova from diethylstilboestrol-exposed mice were capable of fertilization and (3) utilize in-vitro and in-vivo techniques to examine early embryonic development of second generation progeny from such animals.

Materials and Methods

Animals. Virgin CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA, USA), 6–8 weeks of age, were utilized for mating purposes. All animals were housed in a controlled environment which included a 14 h light to 10 h dark cycle (lights on at 06:00 h and off at 20:00 h), food and water *ad libitum* and a temperature of 22°C. Females were placed with individual males and examined daily for copulatory plugs. The presence of a copulatory plug indicated that mating had occurred and was considered Day 1 of gestation. Experimental mice were given a single subcutaneous injection of diethylstilboestrol (Sigma Chemical Company, St Louis, MO, USA) at a dose of 10 µg/kg body weight in 0.1 ml corn oil on Day 15 or 16 of gestation. These gestational times were selected because at these times oestrogen receptors appear in the developing reproductive tract (Holderegger & Keefer, 1986) and most of the developing oogonia are entering prophase of the first meiotic division (Odor & Blandau, 1969) and may be susceptible to mutagenic effects. The dosage of diethylstilboestrol had previously been shown to cause marked structural alterations in the mouse ovary (Wordinger & Highman, 1984). Control mice received 0.1 ml corn oil without diethylstilboestrol on the identical day of gestation. All animals were allowed to give birth spontaneously and the progeny were examined for gross abnormalities at the time of birth. Offspring were weaned at 4 weeks of age and subsequently housed in groups of 3 until 6–8 weeks of age.

Superovulation. Control and diethylstilboestrol-exposed female progeny at 6–8 weeks of age were induced to ovulate by the intraperitoneal injection of 5 i.u. PMSG (National Pituitary Agency, Baltimore, MD, USA) followed 44 h later by 5 i.u. hCG (Sigma). Injected mice were caged overnight with individual males. Mating was verified the following morning by the presence of a copulatory plug.

Embryo recovery following superovulation. For the collection of ovulated ova, mice were killed by cervical dislocation on the morning when a copulatory plug was detected. At this time, ova are found within the ampulla of the oviduct and are embedded in a cumulus mass (Rafferty, 1970). Oviducts were dissected out and placed into individual drops of Brinster's medium (Brinster, 1968). With the aid of a dissecting microscope, the oviduct wall adjacent to the cumulus mass was torn with fine forceps which allowed the cumulus mass containing ovulated eggs to be expressed into the medium. To count accurately the number of ovulated ova, individual cumulus masses were transferred with a 9-mm Pasteur pipette into a drop of hyaluronidase solution prepared by adding 300 units of bovine testis hyaluronidase (Type VI, Sigma) and 200 mg polyvinylpyrrolidone (PVP) in 20 ml phosphate-buffered saline. After 5 min at room temperature the cumulus cells became detached from the ova and the ova were easily counted.

For the collection of 2-cell embryos, mice were killed 24 h after detection of a copulatory plug. At this time, embryos are found throughout the oviduct but not in a distinct cumulus mass (Rafferty, 1970). Oviducts were removed, a 30-gauge blunted needle was inserted into the fimbria with the aid of a dissecting microscope, and 0.05 ml of Brinster's medium was flushed through each oviduct. The 2-cell embryos from each animal were collected, pooled and counted.

For the collection of blastocysts, mice were killed 96 h after the detection of a copulatory plug. At this time, embryos are found throughout the uterus (Rafferty, 1970). Uteri were removed, a 30-gauge blunted needle was inserted into the anterior portion of each uterine horn, and 0.1 ml of Brinster's medium was flushed through each uterine horn. Blastocysts from each animal were collected, counted and pooled.

In-vitro embryo culture and assessment of development. About 20–30 two-cell embryos were collected from mice and subsequently transferred, using a finely drawn Pasteur pipette, to microdrops (0.1 ml) of Brinster's medium and incubated at 37°C in a continuous-flow gaseous atmosphere of 95% air–5% carbon dioxide. Cultures were examined 48 h later for development to the blastocyst stage.

Then 20–30 blastocysts from each group were transferred to 35-mm sterile Falcon plastic culture dishes (Beckton Dickinson Labware, Oxnard, CA, USA) containing 2.0 ml CMRL-1066 medium (Flow Laboratories Inc., McLean, VA, USA) supplemented with 20% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 1 mM-glutamine, 1 mM-sodium pyruvate, penicillin (100 units/ml) and streptomycin (50 µg/ml). Culture dishes were incubated at 37°C in a continuous-flow atmosphere of 95% air–5% carbon dioxide and left undisturbed for 48 h. Starting at 48 h after blastocyst incubation and continuing at 24-h intervals, the following criteria were utilized to assess normal development *in vitro*: (1) hatching from the zona pellucida, (2) blastocyst attachment to the plastic substratum, (3) formation of trophoblast cell outgrowth, and (4) the formation of a discrete inner cell mass (ICM). All developmental criteria were examined using a Model IMT Olympus inverted research microscope.

Measurement of trophoblast cell outgrowth. Beginning at 48 h after initial blastocyst culture, individual blastocysts which had hatched from the zona pellucida, attached to the plastic substratum, and exhibited trophoblast cell outgrowth were photographed at ×40 magnification with a Model IMT Olympus inverted research microscope using

black and white film. All negatives were enlarged to exactly 8×10 in (20×25 cm). The area of trophoblast cell outgrowth was measured on each photomicrograph with a Model 4000 Graphic Data Image Analyzer (Ladd Research Industries, Inc., Burlington, VT, USA).

Statistical methods. The significance of differences between experimental and control means was determined by Student's *t* test for significance (Steel & Torrie, 1960).

Results

Superovulation and embryo recovery

As shown in Table 1, animals exposed *in utero* to diethylstilboestrol on Day 15 or 16 of gestation exhibited a significant increase in the number of ovulated ova, 2-cell embryos and blastocysts in comparison to control animals. In addition, experimental animals which received diethylstilboestrol on Day 15 of gestation had a greater number of embryos at each developmental stage than did animals exposed on Day 16 of gestation. There was no significant difference in the control animals with regard to the number of recovered 1-cell embryos, 2-cell embryos or blastocysts. The results in Table 1 also indicate that normal 2-cell and blastocyst stage embryos were recoverable from the reproductive tracts of the treated mice. This indicates that fertilization, embryo transport, and early embryo development took place. However, the number of embryos at the various developmental stages declined from the 1-cell to the blastocyst stage. The rate of decline appeared to be greater for mice exposed to diethylstilboestrol on Day 15 than on Day 16.

Table 1. Effect of exposure *in utero* to diethylstilboestrol (DES) on superovulation and embryo recovery in mice

Treatment	Day of pregnancy	1-Cell	2-Cell	Blastocyst
Control	15	26.5 ± 2.3	25.1 ± 2.0	24.6 ± 1.5
DES	15	$54.8 \pm 5.7^{**}$	$42.5 \pm 3.6^{**}$	$40.2 \pm 0.6^{**}$
Control	16	30.5 ± 2.9	30.5 ± 3.1	29.8 ± 0.8
DES	16	$42.2 \pm 3.1^*$	$40.7 \pm 2.9^*$	$37.7 \pm 0.7^*$

Values represent the mean ($\pm$ s.e.m.) of 20–50 mice per developmental stage.

* $P < 0.05$, ** $P < 0.01$ compared with control value.

In-vitro embryo development and trophoblast outgrowth

As shown in Table 2, 2-cell embryos from offspring exposed to diethylstilboestrol *in utero* exhibited normal in-vitro development to the blastocyst stage. There was no significant difference between the development potential of 2-cell embryos recovered from mice exposed to diethylstilboestrol on Day 15 or 16 of gestation when compared to the control group. Essentially all the 2-cell embryos that were cultured reached the blastocyst stage of development. Blastocysts which were recovered after superovulation of mice exposed to diethylstilboestrol on Day 15 or 16 of gestation displayed normal in-vitro hatching from the zona pellucida, and attachment to the plastic culture dish. By 48 h after the start of culture, >90% of the control and experimental blastocysts had hatched from the zona pellucida and had started to attach to the plastic culture dish (data not shown). Mural trophoblast cells grew out from the sites of attachment as a monolayer. These were flattened cells which contained enlarged nuclei that were frequently observed to be binucleate and to contain numerous cytoplasmic vacuoles. Van Blerkom *et al.* (1979) have previously reported that the presence of binucleate cells indicates the transformation of mural trophoblast cells into giant trophoblast cells. The inner cell mass, which will give rise to the embryo proper, remained as a

clump of cells above the surface of the trophoblast cell monolayer. With an increase in the culture time the trophoblast cell monolayer continued to increase in size and to migrate away from the centrally located inner cell mass.

Table 2. Influence of exposure *in utero* to diethylstilboestrol (DES) on in-vitro development of 2-cell embryos to the blastocyst stage in mice

Treatment	Day of pregnancy	2-Cell	Blastocyst
Control	15	26.2 ± 1.4	25.3 ± 0.8
DES	15	25.6 ± 0.8	25.1 ± 0.5
Control	16	29.1 ± 1.1	30.6 ± 0.9
DES	16	28.7 ± 0.9	27.8 ± 0.7

Values represent the mean (± s.e.m.) of 20–30 embryos. Each group contained 5–10 replications.

As shown in Table 3, blastocysts from control and diethylstilboestrol-exposed animals were cultured *in vitro* for up to 96 h. There were no differences between control and experimental groups in the amount of trophoblast cell outgrowth measured at any of the studied time points.

Table 3. Measurement of trophoblast outgrowth (μm^2) in control and diethylstilboestrol (DES) exposed embryos after in-vitro culture for various times

Treatment	Day	In-vitro culture		
		48 h	72 h	96 h
Control	15	15 793 ± 1145.9	19 654 ± 976.1	25 587 ± 1211.4
DES	15	15 456 ± 1267.3	19 432 ± 889.7	25 431 ± 1003.9
Control	16	15 849 ± 1414.3	19 562 ± 941.6	25 513 ± 1179.6
DES	16	15 060 ± 1487.7	19 185 ± 992.5	25 919 ± 1514.7

Values represent the mean (± s.e.m.) of 20–35 embryos per time.

Discussion

There is ample evidence that progeny from mice exposed to diethylstilboestrol develop marked reproductive tract abnormalities as growth and development continue from puberty to adulthood. Abnormalities have been reported in the vagina (Plapinger & Bern, 1979), cervix (Bern *et al.*, 1976), oviduct and endometrium (Wordinger & Morrill, 1985). In addition both prenatal and neonatal exposure of mice to exogenous oestrogens, including diethylstilboestrol, results in adult ovaries that are distinguished by (1) the absence of corpora lutea, (2) the presence of follicles in various stages of growth and atresia, (3) hyperplasia and hypertrophy of the interstitial tissue and (4) the appearance of persistent oestrus (Takasugi & Bern, 1964; Kimura *et al.*, 1967; Forsberg, 1969; Mori, 1979; Wordinger & Highman, 1984). These results seem to indicate that the ovary has been directly altered by diethylstilboestrol exposure or permanent changes have occurred in hypothalamo–hypophyseal functions which influence ovarian function.

Haney *et al.* (1984) and Tenenbaum & Forsberg (1985) showed that exposure of mice to diethylstilboestrol *in utero* resulted in aberrant patterns of ovarian steroids. However, Tenenbaum & Forsberg (1985) also demonstrated that, after transplantation of diethylstilboestrol-exposed ovaries to ovariectomized control females, the steroid pattern changed to that characteristic of the control ovaries. Conversely, control ovaries transplanted to diethylstilboestrol-treated females had a steroid pattern similar to that of ovaries exposed to diethylstilboestrol. The induced ovarian alterations were reversible and therefore ancillary to regulatory mechanisms outside the ovary. These results confirm the hypothesis that the ovaries are not permanently altered by prenatal or neonatal exposure to diethylstilboestrol but rather are responding to an abnormal hormonal environment. The primary defect responsible for the anovulatory syndrome and altered ovarian structure and function would seem to exist at the level of the hypothalamo-hypophysial axis rather than directly at the level of the ovary. Our superovulation results indicate that, even if the developing ovary is directly affected by prenatal exposure to diethylstilboestrol, it is still quite capable of responding to exogenous gonadotrophins and producing normal ova which can be fertilized.

In addition to the question of the site of action of diethylstilboestrol, an equally important question has been raised with respect to the developmental potential of ova from progeny of mice exposed to diethylstilboestrol. Our results demonstrate that ovulated ova from such mice are normal in that they are capable of early embryonic development to the blastocyst stage. Also we have demonstrated normal post-blastocyst development, including *in-vitro* hatching from the zona pellucida, attachment to a substratum, trophoblast cell outgrowth and inner cell mass differentiation. Second generation mouse progeny from diethylstilboestrol-exposed mothers therefore appear to be normal at these early stages of development as measured by *in-vitro* growth. This may also be true in humans. An *in-vitro* fertilization programme reported that women exposed to diethylstilboestrol did not differ in their response to exogenous gonadotrophins from general patients with tubal disease (Muasher *et al.*, 1984). The number and, more significantly, the quality of oocytes recovered from the diethylstilboestrol-exposed women did not differ significantly from the control population.

Collectively, these results suggest that the pregnancy complications reported for women exposed to diethylstilboestrol, such as spontaneous abortions (Herbst *et al.*, 1980; Barnes *et al.*, 1980), ectopic pregnancies (Cousins *et al.*, 1980; Berger & Goldstein, 1980) and premature births (Sandberg *et al.*, 1981; Stillman, 1982), may result from structural and/or functional abnormalities in various regions of the reproductive tract which result in the inability to support normal embryo development rather than ova/embryo defects. In fact, vaginal (Herbst *et al.*, 1972), cervical (Pomerance, 1973), uterine (Kaufman *et al.*, 1977; Haney *et al.*, 1979) and oviducal (DeCherney *et al.*, 1981) alterations have been reported in humans after exposure to diethylstilboestrol. Our results for the mouse are supportive of this hypothesis in that progeny from treated mice produced 2-cell embryos capable of normal *in-vitro* development to the post-blastocyst stage but a decrease in the number of embryos capable of developing to blastocysts *in vivo*. Further information on the viability of second generation progeny from diethylstilboestrol-exposed animals could be obtained with the transfer of blastocysts from exposed progeny to control animals and the reciprocal transfer of control embryos into diethylstilboestrol-exposed progeny.

However, later developmental defects have been seen in second generation progeny from diethylstilboestrol-exposed mice which would not be apparent at the early stages of embryogenesis examined in this study. For example, Walker (1984) demonstrated the occurrence of uterine tumours in the progeny of mice exposed prenatally to diethylstilboestrol. Results of this type have not been reported in the second generation progeny of women exposed to diethylstilboestrol but this population may warrant careful examination.

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