Molecular Human Reproduction vol.6 no.2 pp. 154–162, 2000

Diethylstilbestrol (DES)-induced cell cycle delay and meiotic spindle disruption in mouse oocytes during in-vitro maturation

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Due to the growing amount of data related to the deleterious effects of the synthetic oestrogenic compound, diethylstilbestrol (DES), on the female reproductive system, we tested the potential effects of this compound on mouse oocytes. Controlled time- and dose-dependent in-vitro experiments were carried out on isolated cumulus–oocyte–complexes (COCs) to examine the meiotic spindle assembly and chromosome distribution. α-tubulin, chromosomes and F-actin were labelled and detected by confocal laser scanning microscope. COCs were exposed to varying doses of DES (5–30 μmol/l) from the germinal vesicle (GV) stage to the end of metaphase II (MII) when meiosis I and meiosis II is normally completed. Exposure to DES during meiosis I caused a dose-dependent inhibition of cell cycle progression. In comparison with controls, fewer oocytes reached metaphase I (MI) at low doses (5 μmol/l) of DES, while none of the oocytes reached MI in high doses (30 μmol/l). When COCs were exposed to high doses of DES during meiosis II, fragmentation of first meiotic spindle was detected, whereas lower doses caused loosening of the first and the second meiotic spindles. No microtubular abnormalities were detected either in GV-stage oocytes or in cumulus cells. The above data demonstrate that one mode of action of DES on mouse oocytes is a severe yet reversible deterioration of meiotic spindle microtubule organization during maturation.

Key words: confocal microscopy/diethylstilbestrol/in-vitro maturation/microtubule/spindle

Introduction

Disruption of normal structure and function has been at the centre of toxicological studies focusing on reproductive toxicants for many years. Vast numbers of compounds have been reported to cause detrimental outcome during development of the gametes, fetus or neonate. For example, many man-made or generated chemicals used in household products, including pesticides and plasticizers, pharmaceuticals, and dietary supplements, as well as some naturally occurring substances such as phyto-oestrogens found in plants, are adversely affecting reproductive tract development and function (McLachlan, 1985). In developmental exposures, chemicals usually exert their effects only at a specific time during maturation and differentiation and then they disappear. Moreover, they may display a reversible interaction pattern depending on the type of chemical, duration and most importantly the critical timing of exposure (Can and Albertini, 1997a,b). If the agent ceases before the 'vulnerability interval' ends, the adverse developmental effects might be partially or totally overcome (Can and Albertini, 1997b). Therefore, assessing the developmental toxicity of suspected agents is often problematic and requires several precisely-timed dose-response experiments associated with sophisticated analytical procedures.

The profound effects of synthetic oestrogens, in particular, on the developing reproductive tract have been previously demonstrated by prenatal exposure to diethylstilbestrol (DES) (Herbst and Bern, 1981), a non-steroidal compound functionally (but not structurally) similar to natural oestradiol. Historically, DES had been prescribed to women with high-risk pregnancies to prevent spontaneous abortions and other complications of pregnancy. In following years, many health problems have been reported implicating DES in both female reproductive system abnormalities, e.g. vaginal adenocarcinoma, abnormal pregnancies, reduction in fertility and immune system disorders (see Newbold, 1999 for review). Although DES is no longer used clinically to prevent miscarriage, a major concern remains that when DES-exposed women age and reach the time at which the incidence of reproductive organ cancers normally increase, they will show a much higher incidence of cancer than unexposed individuals. Further, the possibility of second generation effects has been suggested (Turusov *et al.*, 1992; Newbold *et al.*, 1998), which puts still another generation at risk for developing problems associated with the DES treatment of their grandmothers. In emergency situations, e.g. post-coital conceptions, DES is still used as the morning-after pill. Thus, the DES episode continues to be a serious health concern and remains a reminder of the potential toxicities that can be caused by hormonally active chemicals.

Studies of DES-induced adverse effects are primarily focused on its teratogenicity and carcinogenicity. Some in-vivo animal models were suggested (Newbold, 1999) to investigate these effects in which DES was shown to target the ovaries causing series ovarian developmental failures including early depletion of follicles (Sangvai *et al.*, 1997) multi-ovular follicles (Iguchi *et al.*, 1990) and lower implantation rates (Pal *et al.*, 1997). Although several complex mechanisms are involved in ovarian follicular and stromal development and are not easy

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Table I. Experimental design for diethylstilbestrol (DES) exposure of cultured cumulus–oocyte–complexes (COCs)

Culture time (h)	0	8	18
Stage of maturation	Isolation of COCs at GV	Metaphase I	Metaphase II
Control	----------		
Control	----------	----------	----------
DES	————		
DES	----------	————	
DES and recovery	————	----------	

—— = periods of drug exposure; ---- = culture periods in control medium; GV = germinal vesicle.

to differentiate, further approaches would help to elucidate the cellular response to DES. Since in-vivo models do not enable testing to observe whether toxicity occurs due to the impairment of hypothalamic/pituitary axis or by direct action on target tissues/cells, we used a unique 2-cell model to monitor the detrimental effect of DES on oogenesis. In the present model, as we have previously shown (Can and Albertini, 1997a), we screened the meiotic cell division with regard to the formation of meiotic spindle and chromosome distribution during in-vitro maturation of mouse cumulus–oocyte–complexes (COCs). Controlled exposure of COCs to varying doses of DES and during different stages of meiotic progression beginning from the germinal vesicle (GV) stage to the end of metaphase II (MII) enabled the analysis of the effects of DES on meiotic spindle formation and function. The formation of first meiotic spindle was tested during the first 8 h of treatment, whereas the second treatment period of 10 h enabled us to test the effect of DES after the first meiotic spindle had been assembled. The results show that DES is a potent yet reversible disrupter of meiosis in mouse oocyte through actions that perturb both cell cycle progression and microtubular organization.

Materials and methods

Collection and culture of mouse COCs

Ovarian follicular development was stimulated by i.p. injection of 5 IU pregnant mare's serum gonadotrophin (PMSG; Sigma Chemical Co, St. Louis, MO, USA) in 19–21 day-old female Balb-C mice. Animals were killed after 48 h of injection and COCs were collected (n = 1655) from isolated ovaries and by follicular puncture. Upon isolation, they were cultured for different time intervals (see below) in minimal essential medium (MEM; Sigma Chemical Co) supplemented with Earle's salts, 2 mmol/l L-glutamine, 0.23 mmol/l pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 3% bovine serum albumin (BSA) in a humidified atmosphere of 5% CO_2 at 37°C.

DES experiments

Diethylstilbestrol (DES) (purity 99.7%, M_r 268,4) (Sigma Chemical Co) was dissolved in dimethylsulphoxide (DMSO) as a 10 mmol/l stock solution and freshly prepared working solutions of 5, 15 and 30 (in mmol/l final concentrations) were used to treat COCs during different stages of oocyte maturation. DMSO concentration of treated and control samples never exceeded 0.1% v/v and at a given concentration, no adverse effects of DMSO on oocyte maturation were observed.

Three different sets of experiments were carried out using three different doses of DES at specific stages (Table I). One group of COCs was treated during initial stages of in-vitro maturation for 7–8 h between the GV stage and MI. A second group was exposed to DES during an 8–18 h interval between MI and metaphase II (MII). In the final set of experiments, COCs were exposed to low (5 μmol/l) or high doses (30 μmol/l) of DES for 8 h, subsequently washed twice in control medium and then transferred to control fresh media for the remaining 10 h of culture to test the reversibility of DES under these conditions. In all experiments, groups of control COCs were associated; therefore, the meiotic status of oocytes was evaluated immediately before or after the interval of DES exposure.

Fixation and staining of oocytes

After removal of cumulus cells by gentle pipetting, control and treated oocytes were fixed and extracted, for 30 min at 37°C, in a microtubule stabilization buffer (0.1 mol/l PIPES, pH 6.9, 5 mmol/l $MgCl_2$ $6H_2O$, 2.5 mmol/l EGTA) containing 5.4 % formaldehyde, 0.1 % Triton-X 100, 1 μmol/l taxol, 10 IU/ml aprotinin and 50% deuterium oxide (Herman *et al*., 1983), washed three times in a blocking and aldehyde-reducing solution of phosphate-buffered saline (PBS) containing 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 mol/l glycine and 0.01% Triton X-100 and then stored at 4°C until processed.

Multiple fluorescence labelling has been performed to evaluate the organization of meiotic spindle microtubules, microtubule organizing centres (MTOCs), F-actin and chromosomes. For visualization of microtubules, optimal results were obtained using a 1:50 dilution of a rat monoclonal antibody, YOL1:34 (Kilmartin *et al*., 1982), specific for α-tubulin. After treatment with primary antibody, samples were incubated with fluorescein-conjugated anti-rat secondary antibodies (Jackson ImmunoResearch Laboratories, West Group, PA, USA). Primary and secondary antibodies were diluted in blocking buffer (see above) and applied for 90 min at 37°C in a humidified chamber. Filamentous actin was localized by incubating samples for 90 min at 37°C with rhodamine–phalloidin (Molecular Probes, Eugene, OR, USA) at a final concentration of 40 IU/ml in blocking buffer. For the evaluation of chromosome staining at specific steps in meiotic progression, oocytes were stained by 10 mmol/l of 7-aminoactinomycin-D (Sigma Chemical Co). Then oocytes were mounted between glass coverslips and slides using spacers allowing a ~100 μm space in between (Can A., 1996) which was filled with a 1:1 glycerol/PBS medium containing 25 mg/ml sodium azide as anti-fading reagent.

Confocal microscopy

Labelled oocytes were examined and images were recorded using a Zeiss LSM-510 confocal laser scanning microscope (Germany) equipped with 488 nm Argon ion, 543 nm green He–Ne, 633 nm red He–Ne lasers and a ×63 Zeiss Plan-Apo objective. Single and z axis optical sections were collected by LSM-510 Software (Germany) running on a Siemens-Nixdorf PC. The final three dimensional (3D)

Table II. Effects of diethylstilbestrol (DES) on meiotic maturation kinetics. Percentages in each meiotic stage are indicated from a total of 1655 oocytes

Time (h)	Dose (μmol/l)	ProMet (%)	MI (%)	AI (%)	TI (%)	MII (%)	Abnormal
0–8 Control	–	13	72	13	1	1	–
0–8 DES	5	48	38	14	–	–	–
0–8 DES	15	18	21	–	–	–	61
0–8 DES	30	3	–	–	–	–	97
8–18 Control	–	1	4	1	–	94	–
8–18 DES	5	6	21	11	24	38	–
8–18 DES	15	–	31	1	–	–	68
8–18 DES	30	8	3	–	–	–	89
0–18 Control	–	2	5	1	–	92	–
0–8 DES + recovery	5	28	32	4	21	15	–
0–8 DES + recovery	30	6	91	3	–	–	–

ProMet = prometaphase; MI = metaphase I; AI = anaphase I, TI = telophase I; MII = metaphase II.

reconstructions and distance measurements were performed using Zeiss 3D LSM Software (Germany).

Results

Effects of DES on meiotic cell cycle progression

The effects of DES on meiotic cell cycle progression were evaluated by treating COCs for different time intervals during in-vitro meiotic maturation process. COCs were exposed to various doses of DES for either 0–8 or 8–18 h of culture (Table I), during which in-vitro oocyte maturation in mice is normally completed. In all experiments, oocytes were fixed and meiotic stage was determined by analysing the chromosome distribution pattern and F-actin decoration. Results were summarized in Table II in which the percentages in each meiotic stage are indicated.

In general, DES caused a dose-dependent inhibition of cell cycle progression compared to control cells (Table II). Of all isolated GV-stage oocytes, 17% did not resume meiosis in control groups. In none of any DES group was the number of oocytes that remained at GV-stage significantly different from in controls. Therefore, all GV oocytes remained both in control and DES-treated cultures were considered as incompetent to resume meiosis and excluded from the study.

In the 5 μmol/l DES group during the first half of meiotic division (meiosis I), many cells (48%) remained in prometaphase, whereas the rest somehow reached to MI (38%) or even to anaphase I (14%). Compared with controls, this group showed a slight delay in progression and was blocked mostly around prometaphase I. In the 15 μmol/l DES group, only 39% of cells displayed a proper chromosome alignment consistent with either prometaphase I (18%) or MI (21%). The rest of the cells (61%) had irregular and variable chromosome patterns implying a moderate to severe impairment of chromosome alignment in this group. To test the extreme effects of DES in this period, 30 μmol/l of DES was used. In this group, almost all cells (97%) displayed abnormally condensed chromosomal patterns that are not compatible with any of the meiotic stages. Typically in these oocytes a compact mass of chromosomal material was located close to the centre of the cell and a failure to form normal chromosomal bivalents was noted (see Figures 1 and 2, for details). Only a small portion of cells (3%) showed a prometaphase I pattern.

In the second half of meiotic division (meiosis II), where the transition from MI to MII lasts for ~8–10 h in mice, COCs were exposed to DES in the same doses used in meiosis I. In low doses of DES (5 μmol/l), cells reached different stages of maturation ranging from prometaphase I to MII (27%) with minor alterations in chromosome distribution. Compared with controls, DES-treated cells were predominantly found in anaphase I and telophase I during which normal cells could be barely detected. This indicates that the machinery at certain cell cycle check-points is impaired by DES and, therefore, the transition from MI to MII takes longer than in controls. It also implies that DES, directly or indirectly, interacts with cytokinesis, since anaphase and particularly telophase are crucial steps for the accomplishment of asymmetric cell division that results in polar body extrusion. Intermediate doses of DES (15 μmol/l) impaired meiotic progression more significantly, since 31% of cells remained at MI, just a few in anaphase I (1%) while the rest (68%) were diagnosed as abnormal. Those included compaction of chromosome bivalents and occasionally fragmentation of the chromosome set into two pieces implying an improper chromosome separation. High doses of DES (30 μmol/l) caused the most dramatic arrest of meiosis. Few oocytes (11%), in this group, showed normal prometaphase or metaphase figures while the majority of oocytes (89%) arrested during or after prometaphase I. Chromosomes were either located in the centre of ooplasm as a condensed spot or dispersed throughout the ooplasm. The latter finding indicates that the integrity of the first meiotic spindle was totally lost due to DES exposure, since the 'pieces of chromosomes' dispersed into cytoplasm after they had been successfully organized in a bipolar metaphase plane prior to DES exposure.

Effects of DES exposure on spindle formation during MI and MII

After preliminary results showing abnormal redistribution of oocyte chromosomes due to DES exposure, we performed multilabel fluorescent staining to determine whether the effects of DES on meiotic maturation affected spindle microtubule integrity. For this purpose, antibodies raised against total α-tubulin were used to visualize the entire meiotic spindle(s) microtubules elongating from poles to kinetochores, as well as MTOCs.

Control MI spindles displayed the typical barrel shape and were located at the periphery of the cell (Figure 1A). Chromosomes were lined up on mid-plate, where they were tightly bound to kinetochore microtubules. The long axis of spindle which transverses the spindle poles was always seen perpendicular to plasma membrane. The mean distance from pole-to-pole was found to be 26.29 μm in control MI oocytes. Control MII spindles, on the other hand, were seen to be slightly smaller than MI spindles regarding the microtubule mass (Figure 1B) and interpolar distance (22.50 μm), yet they maintained a barrel shape appearance. They were often located close to the polar body and slightly rotated compared with MI

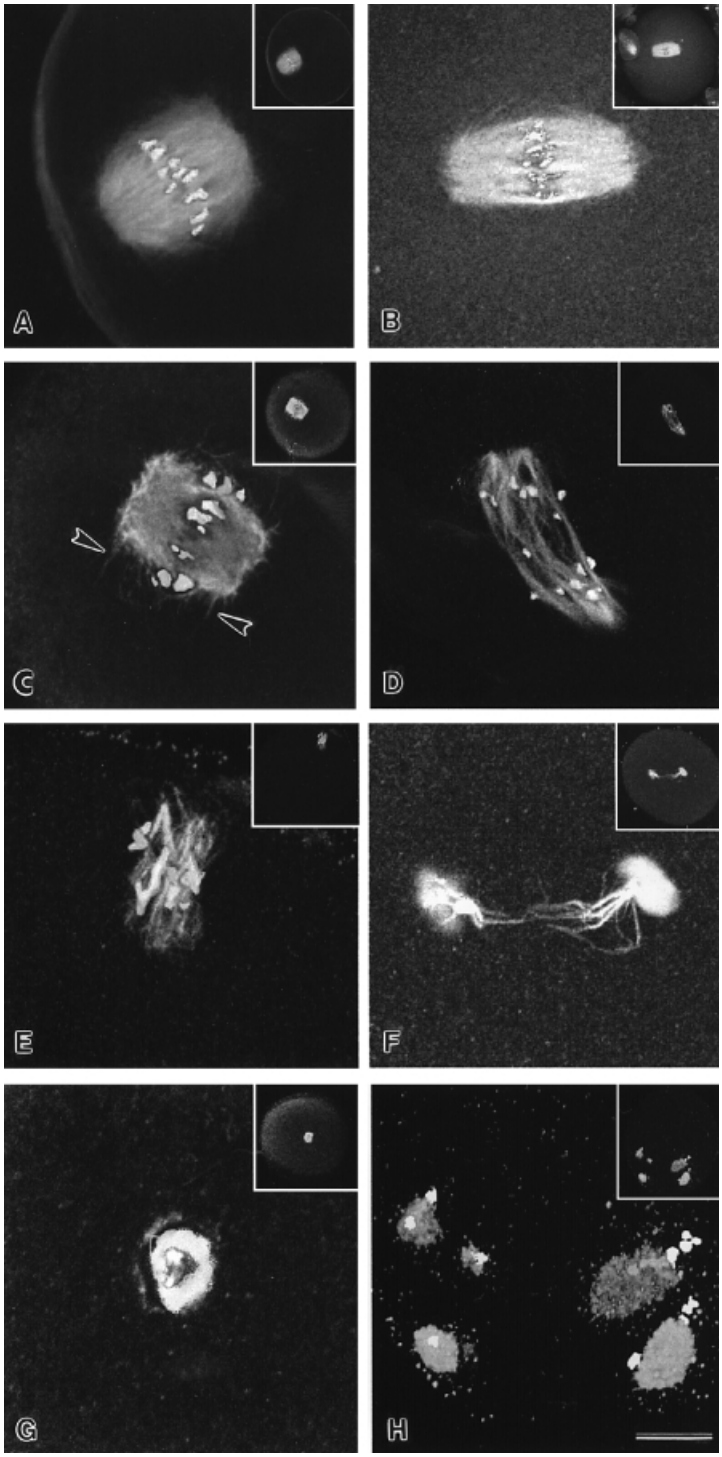

Figure 1. Effects of 5–30 μmol/l doses of diethylstilbestrol (DES) on meiotic spindles and chromosome distribution. Upper right inlets show the location of the spindle in the oocyte. (**A**) Typical barrel shape metaphase I (MI) spindle and (**B**) metaphase II (MII) spindle are seen associated with the lined-up chromosomes at the mid-plane. (**C**) Spindle integrity is impaired due to 5 μmol/l DES, since MI spindle exhibits an expansion both from poles and lateral sides. However, note the displaced microtubules (arrowheads) still attached to the spindle halves either with their polar or kinetochore ends. Loosening and elongation of spindles are further noted in (**D**) an anaphase I spindle due to 5 μmol/l DES during meiosis II. The number of microtubules is low and chromosome migration from the mid-plane to poles is unsynchronized. (**E**) More serious and earlier loosening of spindle is detected in spindles when cells were treated with 15 μmol/l DES. (**F**) A severe example of 15 μmol/l DES exposure is seen when cells were treated after the first meiotic spindle had been formed (see also Figure 2A–C). Extreme examples are seen due to 30 μmol/l DES exposure during (**G**) meiosis I and (**H**) meiosis II. Compaction of microtubules and chromosomes is retained after 30 μmol/l DES during (**G**) meiosis I, whereas the existing MI spindle is fragmented into five unequal pieces, (**H**) as detected by three dimensional (3D) image reconstruction of z axis consecutive confocal images. Bar = 10 μm.

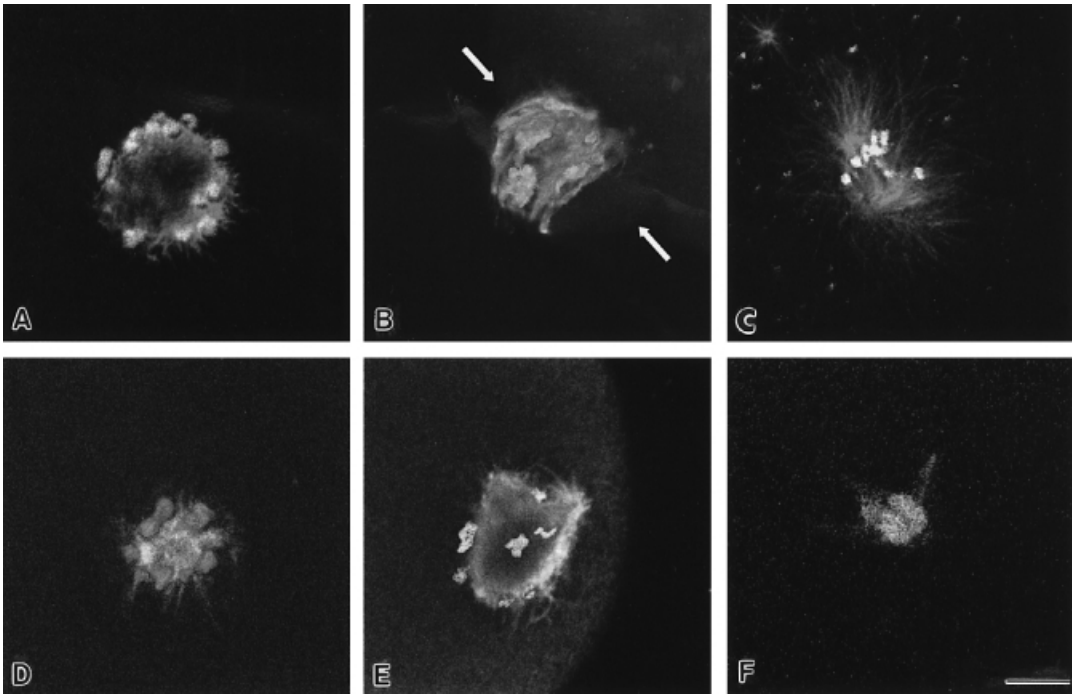

Figure 2. Variations in spindle and chromosome abnormalities due to (**A**–**C**) 15 μmol/l and (**D**–**F**) 30 μmol/l diethylstilbestrol (DES) treatments. (**A**) A prometaphase I figure is seen with a mass of microtubules surrounded by distinct chromosomes. (**B**) After DES exposure during meiosis II, a telophase I figure with a cellular outgrowth (arrows) where the polar body will be extruded; and (**C**) an extremely dispersed MI figure with scattered chromosomes. (**D** and **E**) Compaction of meiotic spindle after 30 μmol/l DES exposure during meiosis I. (**F**) A leftover spindle piece is seen after cells were treated with 30 μmol/l of DES during meiosis II. Bar =10 μm.

spindles, so that the long axis became parallel to plasma membrane. These spindle dynamics are normal events that are required for proper meiotic progression (Albertini, 1992).

In 5 μmol/l DES-treated cells, the prominent feature of the MI spindle was the dilation of spindle mass particularly at the pole regions indicating that spindle poles responsible for the integrity of spindle halves were becoming loosened. Another supporting sign of this result was the displacement of outer spindle microtubules, resulting in a loss in the integrity of spindle laterally (Figure 1C). Consistent with this, few chromosomes had migrated from the mid-plate either towards the poles or outside the spindle region. Apart from this slightly altered MI appearance, 48% of cells displayed normal prometaphase I and 14% anaphase I figures. When oocytes were treated with 5 μmol/l DES during meiosis II, more dramatic alterations appeared regarding the spindle assembly and chromosome distribution. An unusual number of cells was detected in transitional stages from MI to MII. Loosening and elongation of spindles (mean interpolar distance = 37.49 μm) were frequently noted in all spindles (Figure 1D). Tubulin mass was significantly low, evidenced by the loss of many microtubules due to DES exposure. Chromosomes were localized arbitrarily between poles and the mid-plate, a critical point, which may prospectively give rise to the non-disjunction of chromosomes in later stages.

Intermediate doses of DES (15 μmol/l) were administered next to see more severe effects of DES on spindle formation. The profound outcome was the formation of several abnormal oocytes (61%) bearing significantly deteriorated spindles. It was difficult to consider each modified spindle in one group, therefore a series of common malformations is documented in Figure 1E, Figures 2A, B and C from an abnormal prometaphase I (Figure 2A), to MI figures (Figure 1E). The formation of abnormal spindles largely depended on the stage during which a given cell had been maximally exposed to DES. In cells exhibiting MI figures, spindles were found similar to the ones detected in the 5 μmol/l DES group (Figure 1E). When cells were treated after the MI spindle had been assembled, they were totally unable to form the second meiotic spindle and, therefore, failed to extrude polar body. In these examples, extremely scattered spindle microtubule forms were noted (Figures 1F and 2C) some of which were associated with clumps of chromosomes (Figure 1F) rather than individual chromosome bivalents. Measurement of interpolar distance was not applicable to those spindles. Occasionally, a few telophase I figures were recognized by the existence of a cellular outgrowth (Figure 2B) that gives rise to polar body. However, the location of the telophase spindle was not correct which may result in an aneuploid gamete formation.

For testing the extreme doses of DES on cultured mouse oocytes, 30 μmol/l DES was administered before and after the formation of the first meiotic spindle. As expected, a more drastic rate of abnormal oocytes was detected in this group. Almost no proper alignment of chromosomes or spindle microtubules was observed during meiosis I. The most prominent finding was the apparent loss of spindle tubulin that resulted in a total or partial loss of spindles (Figure 1G and Figure 2D). The remaining tubulin mass, if any, was not able to orient the chromosomes properly, therefore most cells contained small masses of compacted tubulin without a fila-

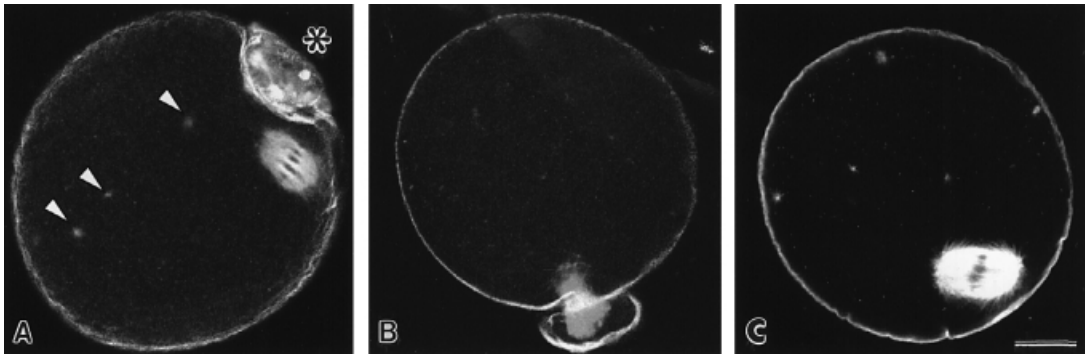

Figure 3. Recovery of oocytes after (**A** and **B**) 5 μmol/l and (**C**) 30 μmol/l diethylstilbestrol (DES). Oocytes are double-labelled with anti-tubulin antibody for microtubules and microtubule organizing centres (MTOCs) with rhodamine-phalloidin for F-actin. (**A**) An intact MII oocyte with a distinct polar body (*). Note the well-organized MTOCs (arrowheads) throughout the ooplasm. (**B**) A telophase I oocyte with a distinct mid-body shared by the oocyte and the polar body. (**C**) An intact MI oocyte recovered from 30 μmol/l DES. Note the reappearance of MI spindle and several MTOCs. Bar = 15 μm.

mentous appearance and intermingled with a clump of chromosomes. Occasionally, distinct chromosome bivalents were noticed after *z* axis 3D image reconstruction renderings (Figure 1G). However, even in these examples, spindle pieces did not have a filamentous appearance, implying that the primary defect was mainly confined to the failure of microtubule assembly consequently giving rise to chromosome misalignment. When cells were treated with 30 μmol/l DES during meiosis II, the existing spindles were consistently fragmented into several small pieces randomly distributed throughout the ooplasm (Figure 1H). Occasionally, severe reduction in the first meiotic spindle microtubules was noted (Figure 2F). The spindle fragments or leftover miniature spindles after DES were most likely dysfunctional, since cells could not extrude any polar body at all.

Recovery of oocytes after DES exposure

To determine whether the effects of DES exposure on cell cycle progression are reversible, oocytes were treated with 5 μmol/l and 30 μmol/l DES for 8 h then washed and transferred to control culture medium for an additional 10 h (see Table I, for experiment design). In 18 h of total culture, most untreated cells progressed to MII (92%). Quite interestingly, a small portion of oocytes (15%) exposed to 5 μmol/l DES were able to progress up to MII (Figure 3A) while a larger portion of cells (25%) accumulated in anaphase I or telophase I (Figure 3B). The remaining 60% of cells were at prometaphase I and MI. In all stages, cells possessed intact spindle microtubules associated with proper chromosome alignment. Cells were only able to reach MI (91%), yet had a normal spindle and well-organized MTOCs (Figure 3C), when recovered from 30 μmol/l DES during meiosis I. No obvious signs of cell death were noted in DES-treated cultures. Taken together, these data show that actions of DES are reversible in a dose-dependent fashion. This is also evidenced by the above observations in which oocytes managed to recover from the cell cycle blocking effect of DES, during the post-dosing period (10 h). However, it seems that recovering cells needed more time to complete meiosis.

Effects of DES on GV-stage oocytes and cumulus cells

During observations of DES-exposed oocytes, few GV stage oocytes and oocyte-enclosing cumulus cells were encountered and served as positive controls for both DES treatments and staining procedures. Interesting, none of the GV stage oocyte or cumulus cell displayed signs of microtubule disassembly or chromatin/chromosome abnormality (Figure 4A). In GV stage oocytes, a well-developed cytoplasmic microtubule network was recognized as delicate bundles of microtubules overlapping each other (Figure 4B). This microtubular lattice was found throughout the ooplasm except where the germinal vesicle is located (Figure 4C). As expected for GV stage oocytes, each non-dividing cumulus cell exhibited well-preserved cytoplasmic microtubules throughout the cytoplasm (Figures 4A and D).

Discussion

The present studies were designed to evaluate the effects of the synthetic oestrogenic compound, DES, on the process of meiotic maturation in cultured mouse oocytes, as a possible model for determining the mode of action of oestrogenic agents on meiosis in mammalian germ cells. Although examining the in-vitro actions of oestrogenic agents on oocytes may not accurately reflect their in-vivo activity, there is mounting evidence to suggest that DES and related compounds impair reproductive function in mammals (Halling and Forsberg, 1990) including human (Pal *et al*., 1997) by a potential action that interferes with meiosis at key stages of germ cell nuclear maturation. In view of the established anti-microtubule effects of DES in purified microtubules (Sharp and Parry, 1985; Sato *et al*., 1987) and in cultured cells (Sakakibara *et al*., 1991), it was anticipated that this agent would disrupt the process of meiotic maturation in mammalian oocytes and would eventually give rise to aneuploidy. Given the multi-step nature of meiosis and the stage-specific involvement of the cytoskeleton in general and microtubules in particular (Albertini, 1992), the present studies were undertaken, for the first time in the literature, to evaluate the actions of DES on oocyte maturation.

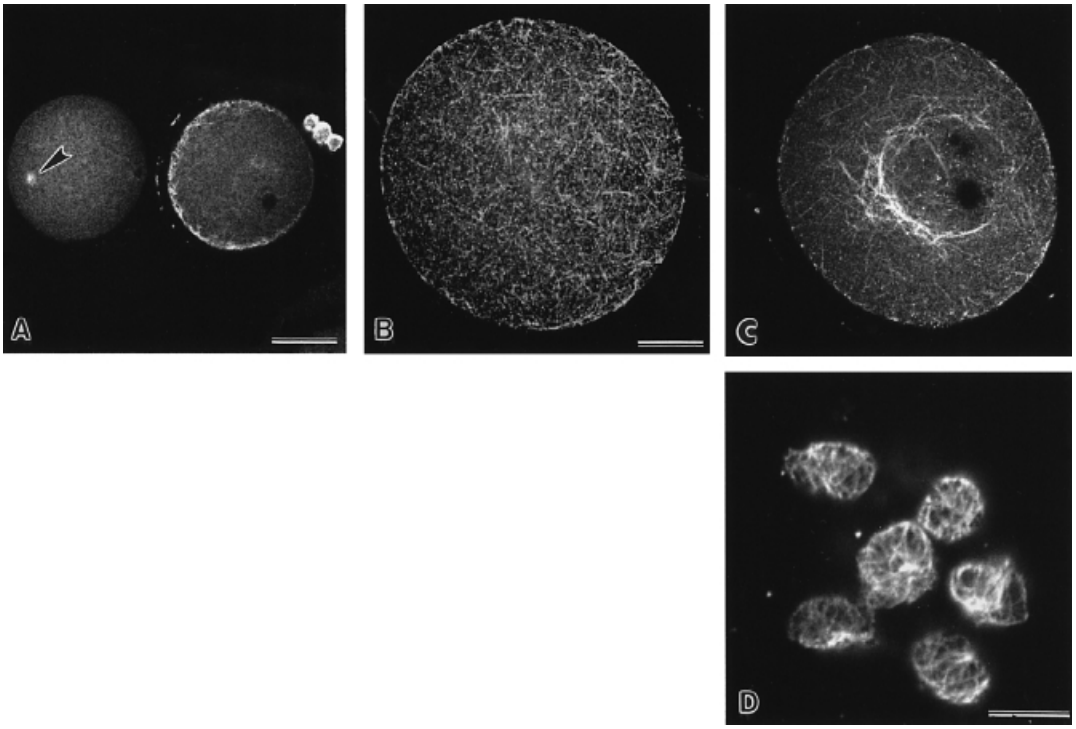

Figure 4. Effects of diethylstilbestrol (DES) on germinal vesicle (GV)-stage oocytes and cumulus cells. (**A**) Intact cytoplasmic microtubules in cumulus cells and a GV stage oocyte next to an abnormal meiotic oocyte consisting of a mass of chromosomes (arrowhead) after exposure to 30 μmol/l DES. (**B** and **C**) Well-preserved cytoplasmic microtubule network throughout the ooplasm encircling the germinal vesicle is resistant to 30 μmol/l DES for 8 h. (**D**) Non-dividing cumulus cells exhibit massive cytoplasmic microtubules still existing even after exposure to 30 μmol/l DES for 8 h. Bars in A = 40 μm; in B and C = 20 μm; in D = 10 μm.

This study shows that DES interferes with the mechanism whose primary function is to build the asymmetric cell division machinery that is unique to mammalian oocytes. Two major consequences of DES exposure have been revealed. First, DES exposure is shown to interfere with meiotic cell cycle progression by delaying advancement to MI and by inhibiting the MI spindle formation during meiosis I. These effects are stage and cell specific since both the initial phase of meiotic maturation, involving GV, germinal vesicle breakdown (GVBD), chromatin condensation and cumulus cells are unaffected even at relatively high concentrations of DES. Secondly, severe disturbances in chromosome alignment are observed that are most likely related, in an indirect way, to the effects of DES on the maintenance of spindle structure, as has been observed by others (Tsutsui and Barret, 1997). Collectively, those results led us to conclude that the microtubule-depolymerizing effect of DES is somehow restricted to meiotic microtubules, at least in tested doses. The resistance of interphase microtubules found either in GV-stage oocytes or cumulus cells suggests that meiosis is more susceptible to the adverse effects of DES and, therefore, might ultimately result in a series of genetic defects in oocytes, e.g. aneuploidy or fertilization failure without grossly altering the surrounding cells and tissues.

The COC is an excellent 2-cell system for investigating the physical and functional relationship between the oocyte and the surrounding cumulus cells. The reason that COCs were used instead of denuded oocytes in present experiments is to simulate the in-vivo conditions to some extent, a closed 2-cell system where an exogenous agent should trespass the first checkpoint (i.e. cumulus cells) in order to reach the enclosed oocyte mainly by the route of transzonal processes and intercellular junctions. The latter are known to provide a dynamic intercommunication (Buccione *et al*., 1990) presumably facilitating two-way trafficking of metabolic products and reagents. The recovery of DES-exposed cumulus-enclosed oocytes, as presented in this study, support this assumption. However, DES-exposed denuded oocytes could not be reversed from abnormal meiotic stages, then one could postulate that the cumulus cells play a regulatory role in the uptake and discharge of environmental agents.

Effective concentrations (EC) of DES required for induction of microtubule disruption in 50% of cells in two breast cancer cell lines have been reported as 48 and 50 μmol/l (Aizu Yokota *et al*., 1994), whereas the lethal dose at which 50% of cells are no longer viable (LD_{50}), was reported as 19–25 μmol/l in prostate cancer cells (Robertson *et al*., 1996). An increase in the number of apoptotic nuclei was detected when the same cells were treated with 15 or 30 μmol/l DES. On the other hand, the in-vivo dose for DES was reported to be 1–5 μg daily when injected in neonatal or 10 day old rats and mice (Halling and Forsberg, 1990; Iguchi *et al.*, 1990). Since the form and duration of exposure, and even the animals' age, are potential variables for the acute toxicity of DES, it is difficult to compare in-vivo and in-vitro doses. Taken together, it is likely that the optimum effective but not lethal dose for in-vitro DES treatment depends on the type of cell and the stage of cell cycle, at least in isolated and cultured cells.

Higher concentrations of DES led to progressive and differential alterations in the organization of both MI and MII meiotic spindles. Despite causing a dramatic decrease in overall microtubule mass in meiosis I, during which the first meiotic spindle is formed, loss of spindle pole integrity dominates during meiosis II, where the existing MI spindle undergoes fragmentation to form several groups of microtubules and chromosomes. The characteristic miniature spindle fragments observed following exposure to 30 μmol/l DES points further to an action of this compound on spindle pole components that must in part be related to the organization and function of centrosomal material. One explanation for this structural effect could be direct interference with α-tubulin, the tubulin isoform known to be exclusively situated within MTOCs or centrosomes (Joshi, 1994). These results collectively support concerns on the role of DES as a causative agent of aneuploidy in mammalian oocytes.

In view of its purported microtubule depolymerizing activity, it was somewhat surprising that microtubule depolymerization was not observed in non-dividing somatic cumulus cells and more interestingly in GV-stage oocytes. It is noteworthy that differences in the selective impairment of spindle microtubules in response to DES could be due either to cell cycle specific alterations in microtubule dynamics (Kuriyama and Borisy, 1981; Robertson *et al*., 1996) or the concentration of the drug used. Mitotic spindle microtubules vary in their susceptibility to different anti-microtubule drugs. For instance, while vinblastin, colchicine or nocodazole depolymerize all microtubule subtypes (Jordan *et al*., 1992), MBC, a commonly used fungicide, exhibit selective disruption of astral and interpolar spindle microtubules in mitotic cells without showing deleterious effects on interphase or kinetochore microtubules (Can and Albertini, 1997b). One possible explanation for the selective impairment of microtubules is a difference in mode of action between several compounds because of differences in cellular uptake or in their conversion to active metabolites, which may have altered binding activity to tubulins. Participation of two distinct forms of tubulin dimers (α and β) in microtubule assembly could explain the differential impairment of certain microtubule populations due to various agents. For instance, a post-transitional change of α-tubulin, e.g. acetylation, has been shown to cast stable microtubules that are significantly resistant to depolymerising agents (Salmon *et al*., 1984). Furthermore, it was demonstrated that differences in acetylation rate of different microtubule populations in a meiotic spindle alter the turn-over rate of microtubule assembly (Webster and Borisy, 1989) which in turn makes meiotic microtubules more susceptible to agents. Diverse microtubule responses to several oestrogenic agents, e.g. DES, 17β-oestradiol, E-dienestrol, bisphenol-A or to their metabolitic components, such as diethylbestrol oxide and indenestrol-A, examined *in vivo* and *in vitro* give support to the dynamic instability model of microtubule polymerization, a phenomenon in which minute changes in time or concentration derive critical outcomes.

Since a hormone receptor–second messenger system is known to be the most common pathway of steroid hormone efficacy, it is possible that cell cycle delay in oocytes due to DES exposure is a receptor-mediated action. However, studies on the microtubule disrupting effect of DES via oestrogen receptors failed to demonstrate that oestrogen receptor-positive cells were more prone to DES-induced toxicity (Aizu Yokota *et al*., 1994). Supporting evidence comes from a microbial study testing the effect of indenestrol-A and B, well-known DES metabolic products, on the oestrogen receptors (Metzler and Pfeiffer, 1995). This study showed that the cytotoxic activity of those end-products was a result of a direct action on microtubule polymerization rather than a receptor-mediated activity, although indenestrols are known to have strong binding affinities for oestrogen receptors. Moreover, this study also demonstrated that, in cell-free systems, the ability of oestrogenic substances to interact with microtubules was not correlated with hormonal activity. The in-vitro maturation model used in this study lends credence to the finding that no hormonal milieu was used in our culture media to mimic the maturation process, suggesting that DES toxicity is independent from the hormonal environment of oocytes. However, future research should be of interest on tracking the DES-binding receptors and target molecules including other cytoskeletal proteins.

The observation that meiotic cell cycle progression was delayed at low DES doses and that most cells arrest at anaphase and telophase in meiosis I, suggests that DES acts at this critical juncture of meiosis. In both mitotic and meiotic systems, anaphase is known to depend on the ubiquitin-mediated proteolysis of cyclins, the regulatory subunit of maturation-promoting factor (MPF) (Murray and Hunt, 1993). DES could prevent the resumption of anaphase or telophase by inhibitory cyclin degradation. This is a plausible explanation for the action of DES since protease inhibitors have been shown to arrest somatic cells at metaphase by inhibiting cyclin degradation (Sherwood *et al*., 1993). It has been shown that compounds arrest mitosis by activation of a mechanism that detects errors in spindle assembly (Murray and Hunt, 1993). Conditions that influence microtubule stability, spindle pole function, or microtubule motors have been shown to cause mitotic arrest in yeast due to a negative feedback control on M-phase progression. The present studies have shown that DES influences both microtubule stability and spindle pole organization in mouse oocytes making it likely that meiotic cell cycle impairment in this system is also subject to negative feedback regulation. Clearly, further studies on cyclin proteolysis and spindle organization will be needed to ascertain the mechanisms whereby DES exerts its effects on mouse oocytes.

Acknowledgements

We thank Professor David F.Albertini for providing the YOL 1:34 antibody. This study was financially supported by NATO-CRG 951282 to AC for his travel expenses, by TUB0TAK-SBAG AYD 164 and 240, Ankara University Research Fund 98090009 for consumables and by DPT 97K120560 and DPT 98K120730 for the assembly and advancement of confocal microscopy unit.

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Received on July 28, 1999; accepted on November 4, 1999