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17β-Estradiol and environmental estrogens significantly affect mammalian sperm function*

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BACKGROUND: Compounds with estrogenic activity can affect reproductive function in mammals. This study investigated possible effects of 17β-estradiol (E_2) and three weakly estrogenic environmental estrogens on mammalian sperm capacitation and fertilizing ability *in vitro*. METHODS: Uncapacitated and capacitated mouse sperm suspensions were incubated for 30 min in the presence of E_2, genistein (Gen), 8-prenylnaringenin (8-PN) and nonylphenol (NP), and then assessed using chlortetracycline (CTC) fluorescence analysis. In addition, treated uncapacitated sperm suspensions were tested for changes in fertilizing ability. RESULTS: In uncapacitated cells, E_2 at ⩾1 μmol/l and Gen, 8-PN and NP at ⩾0.001 μmol/l, significantly stimulated capacitation and acrosome reactions. Hydroxytamoxifen (an estrogen antagonist) did not inhibit responses to any of these compounds. In capacitated cells, E_2 had no effect, but the other three compounds significantly stimulated acrosome reactions. Added to uncapacitated suspensions, 10 μmol/l E_2, 0.1 μmol/l Gen and 0.1 μmol/l 8-PN all significantly stimulated sperm fertilizing ability (~76% oocytes fertilized) compared with untreated control sperm (~36%). CONCLUSIONS: This study provides the first evidence that E_2 and environmental estrogens can significantly stimulate mammalian sperm capacitation, acrosome reactions and fertilizing ability, with the environmental estrogens being much more potent than E_2. The inability of hydroxytamoxifen to block these responses suggests that classical estrogen receptors may not be involved. Whether these responses have effects on fertility *in vivo* remains to be determined, along with the mechanisms of action involved.

Key words: capacitation/fertilizing ability/genistein/nonylphenol/8-prenylnaringenin

Introduction

For many decades, estrogens have been considered to be primarily female hormones, contributing to female health and fertility. However, more recently estrogens have also been shown to play important roles in males (Korach *et al*., 1996; Hess *et al*., 1997; Luconi *et al*., 2002). Estrogen receptors are present throughout the male reproductive tract, and estrogen-receptor α knockout ($ER\alpha^{-/-}$) males become infertile primarily due to malfunctioning of the efferent ductules of the epididymis, with consequent gross disruption of the seminiferous tubules (Hess *et al*., 1997). Aromatase activity (critical for the conversion of androgens to estrogens) occurs in both Leydig and Sertoli cells (Carreau *et al*., 1999), and mice deficient in aromatase show an arrest of early spermiogenesis (Robertson *et al*., 1999). Sperm are exposed to estrogens within the male tract, and P450 aromatase has also been identified in rat sperm cytoplasmic droplets (Hess, 2000) and human sperm flagella (Aquila *et al*., 2002). This raises the possibility that sperm can provide a continuing local source of estrogens in the epididymis and in the female tract. In the latter, sperm would also be exposed to estrogens, particularly in tubal fluid following follicle rupture and when in close vicinity to ovulated oocytes.

The most potent estrogen is 17β-estradiol (E_2), and until recently responses to E_2 were thought to be genomic, with mediation via the nuclear estrogen receptors α and β (ERα, ERβ; Levin, 2001). However, 'non-genomic' effects of estrogens have also been characterized in several cell types (Kelly and Levin, 2001), including those from the reproductive system (Revelli *et al*.., 1998) and possibly sperm (Luconi *et al*., 1999, 2001). These effects are mediated via either membrane-bound receptors or interaction with other proteins and/or membrane lipids (Levin, 2001). Earlier studies using [^{3}H]-E_2 showed that E_2 binds to the sperm surface (Hernandez-Perez *et al*., 1979; Cheng *et al*., 1981b) and suggested that human sperm lack cytosolic and nuclear estrogen receptors (Cheng *et al*., 1981a). Immunohistochemical detection of estrogen receptors has been reported for human (Misao *et al*., 1997; Durkee *et al*., 1998) and rat sperm (Saberwal *et al*., 2002), with distribution in both the head and flagellum. Recently, a putative novel estrogen receptor in human sperm membranes has been

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partially characterized (Luconi *et al*, 1999), although others (Saberwal *et al*., 2002) presented Western blotting data for the presence of ERα in rat sperm.

The estrogen receptor appears to be quite promiscuous in ligand binding (Anstead *et al*., 1997; Blair *et al*., 2000), and many non-steroidal compounds in the environment have been found to possess estrogenic activity (Sohoni and Sumpter, 1998; Akingbemi and Hardy, 2001). Humans are exposed to natural phytoestrogens such as genistein (Gen; found in soy and legumes) (Mazur and Adlercreutz, 2000) and 8-prenylnaringenin [8-PN; found in hops (*Humulus lupulus*) and beer (Milligan *et al*., 1999, 2002)] in the diet. Synthetic chemicals with estrogenic activity include alkylphenol polyethoxylates, such as nonylphenol (NP), that are used in cleaning products, paints, herbicides, pesticides, industrial aids in pulp, paper production and textile manufacturing (Sonnenschein and Soto, 1998). The surfactant nonoxynol is used as an intravaginal spermicide and condom lubricant, and can be metabolized to free NP. Environmental estrogens have the potential to act as endocrine disruptors and so exert negative developmental and reproductive effects in wildlife. Moreover, it has also been suggested that these compounds may be responsible for a variety of reproductive disturbances in men, including possible declines in sperm concentration (Sharpe, 2001; Skakkebaek *et al*., 2001). However, because of their great structural diversity, the modes of action of environmental estrogens are controversial (Cassidy and Milligan, 1998; Ashby, 2001).

In view of the role of endogenous estrogens in the male, and the ability of environmental estrogens—when used at relatively high concentrations—to disrupt spermatogenesis and hence reduce male fertility, it is important to determine whether any of these compounds can have more subtle, direct effects on function in mature sperm, e.g., fertilizing ability. At the time of ejaculation or release into culture medium *in vitro*, sperm are unable to fertilize (Yanagimachi, 1994). However, after a species-dependent time in an appropriate environment either *in vivo* or *in vitro*, sperm acquire the capacity to fertilize and are said to be 'capacitated' (Austin, 1951, 1952; Chang, 1951). Capacitated sperm are able to express hyperactivated motility, to undergo the acrosome reaction, and to fertilize an oocyte (Yanagimachi, 1994). If estrogens are able to affect these events, there could be important consequences for fertility *in vivo.*

Therefore, the present study examined the effects of E_2 and three compounds of proven estrogenic activity, namely Gen, 8-PN and NP, on both uncapacitated and capacitated mouse sperm, looking for any significant effects on the rate of capacitation *per se*, the acrosome reaction and fertilizing ability. [A short abstract of this study was published in the abstract book of the 18th Annual Meeting of the European Society of Human Reproduction and Embryology (Adeoya-Osiguwa *et al*., 2002).]

Materials and methods

Media and reagents

Throughout this study, sperm were suspended in a modified Tyrode's medium containing 1.8 mmol/l calcium chloride (Fraser, 1993) with 4 mg/ml bovine serum albumin (BSA, crystalline; Sigma Chemical Co. Ltd, Poole, Dorset, UK). All chemicals were purchased from Sigma unless otherwise specified. A stock solution of 100 mmol/l E_2 was prepared in dimethyl sulphoxide (DMSO) and stored at –20°C. Working stock solutions were prepared daily by first diluting the initial stock 1 in 10 with DMSO:0.9% NaCl (1:1); subsequent dilutions used standard medium. Stock solutions (10 mmol/l) of Gen, 8-PN and NP were prepared in absolute ethanol (AnalaR grade; Merck Ltd, Poole, Dorset, UK) and stored at –20° C; working stock solutions were prepared daily using standard medium as diluent.

Preparation of sperm suspensions

The contents of cauda epididymides from mature TO male mice (Harlan OLAC, Bicester, UK) were released into 30 mm sterile culture dishes (Nunc, Roskilde, Denmark) containing standard medium (1 ml per two epididymides). For experiments using uncapacitated sperm, suspensions were left for 5 min on a warming tray to allow sperm dispersal, then passed through short columns containing medium-grade G-25 Sephadex beads (Pharmacia, Uppsala, Sweden) to separate motile from non-motile cells. The resulting filtrates, consisting of motile uncapacitated sperm, were used immediately. When capacitated sperm suspensions were used, unfiltered suspensions were allowed to disperse and then incubated for 90 min under autoclaved liquid paraffin (Boots, Nottingham, UK) at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The suspension was then filtered immediately prior to use.

Although a dye such as Hoechst 33258 may be used to determine the live/dead status of human sperm (Green *et al*., 1996), important physiological reasons preclude its use with mouse sperm. The technique involves incubation of suspensions with dye for a few minutes, then centrifugation through polyvinylpyrrolidone to remove free dye. Such a centrifugation step removes decapacitation factor molecules from the surface of mouse sperm, so stimulating capacitation and hence changing the physiological state of the cells (Fraser, 1984), irrespective of the experimental treatment being investigated. For this reason, mouse sperm suspensions were filtered to remove non-motile, possibly dead, cells prior to the short experimental incubation and assessment.

Chlortetracycline fluorescence assay

The chlortetracycline (CTC) fluorescence assay was carried out as described previously (Green *et al*., 1994). In brief, after experimental treatment, sperm suspensions were mixed well and 45 µl of each suspension was added to an equal amount of CTC (750 µmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteine, 20 mmol/l Tris-HCl, pH 7.8) and mixed well. Cells were then fixed by adding 8 µl of 12.5% (w/v) paraformaldehyde in 0.5 mol/l Tris-HCl (pH 7.4). CTC was prepared fresh daily and kept at 4° C, wrapped in foil to exclude light, until needed; aliquots were allowed to warm up before use. Under yellow sodium illumination to minimize fading of CTC, slides were prepared by placing 10 µl of fixed suspension on a clean slide and mixing thoroughly with one drop of 0.22 mol/l 1,4-diazabicyclo [2.2.2] octane (DABCO) dissolved in glycerol: phosphate-buffered saline (PBS) (9:1) to prevent fading of the fluorescence. After adding a coverslip, the slide was compressed firmly between tissues to remove excess fluid and flatten the cells to allow accurate evaluation. To avoid evaporation, the slides were sealed with colourless nail varnish and stored, wrapped in foil, in the cold until evaluation was carried out.

The assessment of sperm was performed using an Olympus BX40 microscope equipped with phase-contrast and BX-FLA epifluorescence optics. The ultraviolet excitation cube was used, with the excitation beam passing through a 400–440 nm band pass filter; CTC was observed through a DM 455 dichroic mirror. In each treatment,

100 sperm cells were assessed and classified as having of one of three staining patterns: (a) F, with bright fluorescence over the entire sperm head; characteristic of uncapacitated, acrosome intact sperm; (b) B, with a fluorescence-free (dark) band in the post-acrosomal region, characteristic of capacitated, acrosome-intact sperm; (c) AR, with dull or absent fluorescence throughout the sperm head; characteristic of capacitated, acrosome-reacted sperm. The presence or absence of the acrosomal cap was confirmed using phase-contrast microscopy.

In-vitro fertilization

Mature female TO mice were induced to superovulate by intraperitoneal injections of 7.5 IU pregnant mare's serum gonadotrophin (PMSG) (Folligon®; Intervet, Cambridge, UK) followed 48–54 h later by 5 IU hCG (Chorulon®; Intervet). Approximately 14–15 h post-hCG, cumulus masses were released from oviducts into standard medium covered with liquid paraffin. Sperm suspensions were prepared as described previously (but were not filtered); after allowing 5 min for dispersal, they were divided into four aliquots and treated as follows: (1) control (untreated); (2) 10 μmol/l E_2; (3) 0.1 μmol/l Gen; (4) 0.1 μmol/l 8-PN. All suspensions were pre-incubated for 15 min, then diluted 1 in 10 into either standard medium or the medium used for pre-incubation; 400 μl droplets from each sample were prepared under liquid paraffin and cumulus masses were added. Dishes were gassed and then incubated at 37°C for 60 min. Oocytes were transferred to fresh droplets of standard medium and then fixed 15 min later with buffered formalin (4% formaldehyde in PBS). After 30 min fixation, oocytes were stained with 0.75% aceto-orcein, mounted on clean slides, and then assessed. Oocytes were considered to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head (Fraser, 1993).

Statistical analysis

The data were analysed using Cochran's modification of the χ^2-test (Snedecor and Cochran, 1980) using a Microsoft Excel program for Windows 98. This test compares responses within replicates; for a significant difference to be obtained, a consistent and reasonable magnitude is required between control and treated sample.

Results

Series I: E_2 promotes capacitation in uncapacitated sperm

Uncapacitated sperm were incubated in the absence or presence of 0.01–100 μmol/l E_2 for 30 min at 37°C (n = 5 for all treatments). A concentration-dependent stimulation of capacitation was observed, with significantly more B pattern cells (capacitated, acrosome-intact) being observed in suspensions incubated with E_2 at 1–100 μmol/l, compared with untreated controls; maximal responses were obtained with 10 and 100 μmol/l (Figure 1). Furthermore, E_2 at 1–100 μmol/l also significantly stimulated acrosome loss, compared with untreated control cells.

Although motility was not evaluated using objective measurements, E_2 had a positive effect on motility, as determined subjectively: more sperm incubated in the presence of E_2 were motile and they exhibited more vigorous motility than cells in untreated control suspensions.

Series II: Environmental estrogens promote capacitation in uncapacitated sperm

Aliquots of uncapacitated sperm suspensions were incubated in the absence or presence of 0.001–1 μmol/l Gen, 8-PN and NP. Since it was not possible to examine all concentrations of all compounds in a single experiment, each concentration was tested in at least three separate replicates (n = 6 for controls; n = 3–6 for experimental treatments). Each compound significantly stimulated capacitation and the acrosome reaction over the whole range of concentrations evaluated. Of the three compounds evaluated, Gen and 8-PN appeared to be more potent than NP, with strong responses being seen with concentrations ⩾0.01 μmol/l Gen (Figure 2) and 8-PN (Figure 3) compared with ⩾0.1 μmol/l for NP (Figure 4). Like E_2, these compounds had a stimulatory effect on sperm motility, with more motile cells and more vigorous motility than in control suspensions.

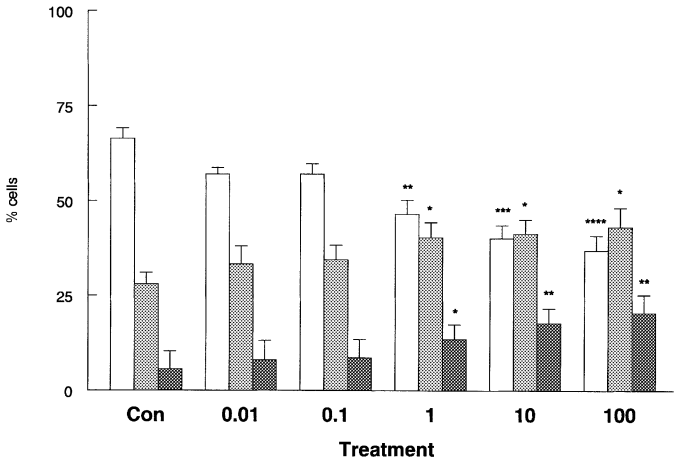

Figure 1. 17β-Estradiol (1–100 μmol/l) significantly stimulated capacitation and the acrosome reaction in uncapacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 5) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. *P < 0.05, **P < 0.025, ***P < 0.01, ****P < 0.001 versus untreated control suspensions (Con).

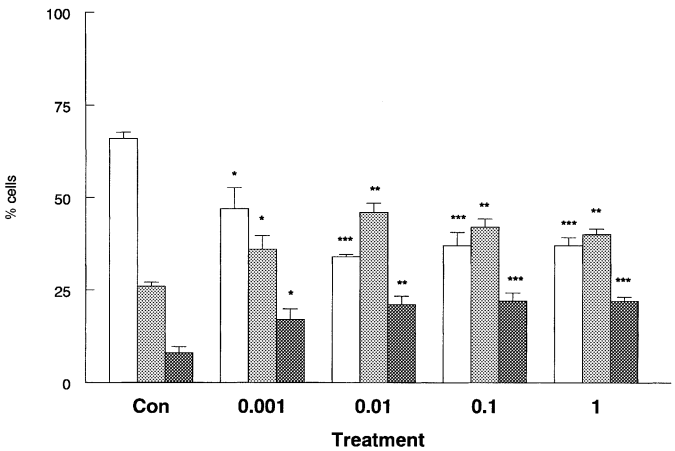

Figure 2. Genistein (0.001–1 μmol/l) significantly stimulated capacitation and the acrosome reaction in uncapacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 3–6) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. *P < 0.05, **P < 0.025, ***P < 0.01 versus untreated control suspensions (Con).

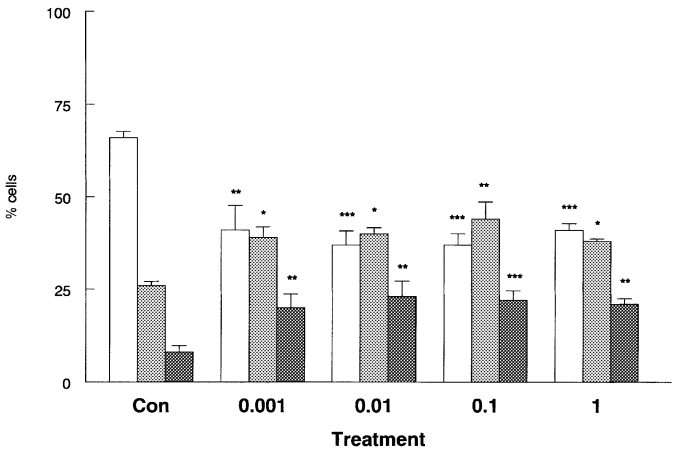

Figure 3. 8-Prenylnarigenin (0.001–1 μmol/l) significantly stimulated capacitation and the acrosome reaction uncapacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 3–6) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. *P < 0.05, **P < 0.025, ***P < 0.01 versus untreated control suspensions (Con).

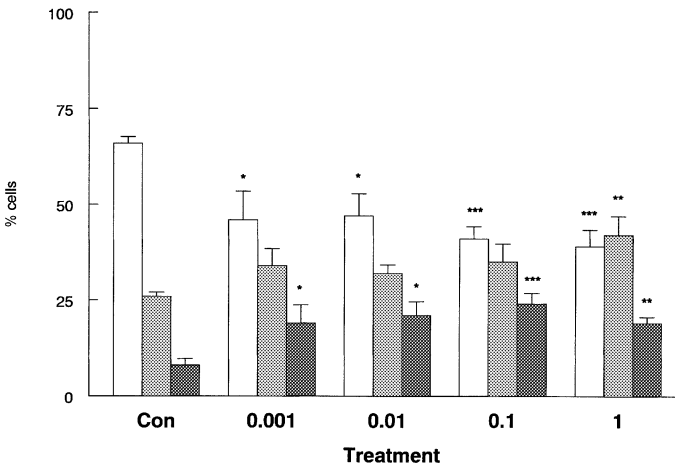

Figure 4. Nonylphenol (0.001–1 μmol/l) significantly stimulated capacitation and the acrosome reaction in uncapacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 3–6) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. *P < 0.05, **P < 0.025, ***P < 0.01 versus untreated control suspensions (Con).

Series III: E_2 has no significant effect on capacitated sperm

In these experiments, sperm were capacitated as described previously and then incubated in the absence or presence of 0.01–10 μmol/l E_2 (n = 4 for all treatments). Although E_2 had very significant effects on uncapacitated sperm (see Figure 1), there were no striking responses in capacitated cells (Figure 5). There was a slight increase in the proportion of sperm expressing the capacitated B pattern, and a slight decrease in the proportion of acrosome-reacted cells in suspensions treated with 1 and 10 μmol/l E_2, compared with the untreated controls, but these differences were not statistically significant.

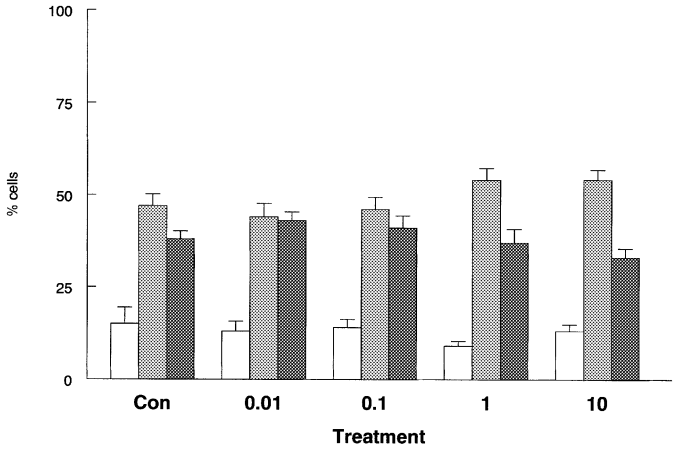

Figure 5. 17β-Estradiol (0.01–10 μmol/l) had no significant effect on capacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 4) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence.

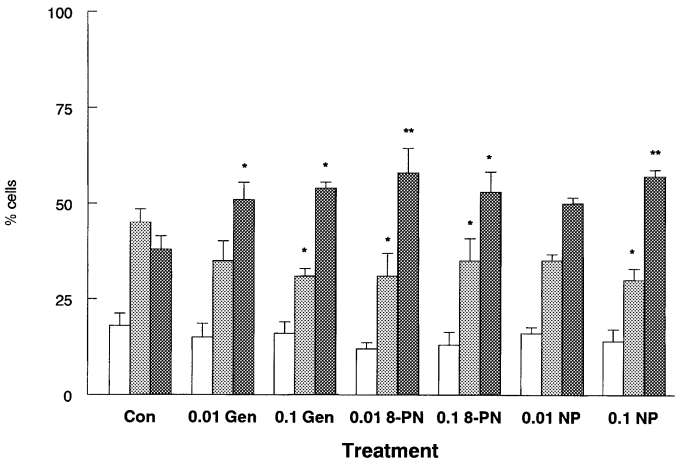

Figure 6. Genistein (Gen) and 8-prenylnaringenin (8-PN) (0.01 and 0.1 μmol/l respectively) and nonylphenol (NP) (0.1 μmol/l) significantly stimulated the acrosome reaction in capacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; n = 4) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. *P < 0.05, **P < 0.025 versus untreated control suspensions (Con).

Series IV: Environmental estrogens stimulate acrosome reactions in capacitated sperm

Capacitated suspensions were prepared and aliquots from the same suspensions were incubated in the absence or presence of 0.01–0.1 μmol/l of Gen, 8-PN and NP (n = 4 for all treatments). There were no significant differences in the proportion of uncapacitated F-pattern cells among untreated control and treated suspensions. However, all three estrogenic compounds stimulated the acrosome reaction, leading to significantly more AR pattern cells, compared with controls, and a corresponding significant decrease in the proportion of capacitated, acrosome-intact B pattern cells. Gen and 8-PN had a significant effect at both concentrations tested; although NP appeared to elicit a response when used at both concentrations, this was only significant with 0.1 μmol/l NP (Figure 6).

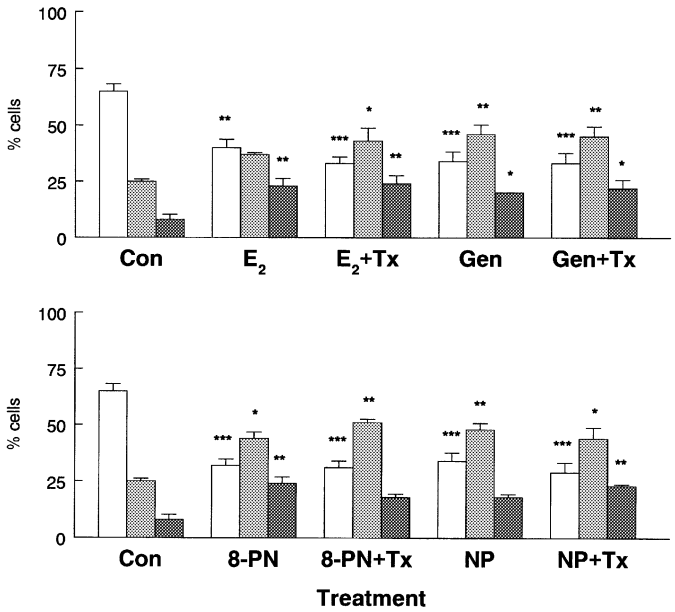

Figure 7. Hydroxytamoxifen (Tx), used at 5 μmol/l, did not inhibit responses to 10 μmol/l 17β-estradiol (E_2) or 0.1 μmol/l genistein (Gen), 8-prenylnaringenin (8-PN) and nonylphenol (NP) in uncapacitated mouse sperm suspensions treated for 30 min, then analysed using chlortetracycline (CTC). Data presented as % cells (mean ± SEM; $n = 3$) expressing F pattern (open bar), B pattern (grey bar) and AR pattern (black bar) of CTC fluorescence. $^{*}P < 0.05$, $^{**}P < 0.025$, $^{***}P < 0.01$ versus untreated control suspensions (Con). To allow easy comparison, the values for the control suspensions are shown in both upper and lower graphs.

Series V: Hydroxytamoxifen does not inhibit responses to E_2 and environmental estrogens in uncapacitated sperm

To determine whether E_2 and the environmental estrogens might be acting via different pathways, hydroxytamoxifen (an estrogen antagonist) was used. Aliquots of uncapacitated sperm suspensions were treated with an estrogenic compound with or without 5 μmol/l hydroxytamoxifen ($n = 3$ for all treatments). Hydroxytamoxifen failed to inhibit responses to 10 μmol/l E_2, 0.1 μmol/l Gen, 0.1 μmol/l 8-PN and 0.1 μmol/l NP (Figure 7); indeed, hydroxytamoxifen appeared slightly to enhance responses to E_2 by promoting the F-to-B transition, though this was not statistically significant.

Series VI: E_2 and environmental estrogens stimulate in-vitro fertilizing ability

The CTC results obtained in Series I and II, indicating a significant stimulation of capacitation, suggested that all four estrogenic compounds would stimulate fertilizing ability *in vitro*. This hypothesis was tested by undertaking in-vitro fertilization experiments using sperm suspensions that had been pre-incubated for ~15 min with no additions (control), 10 μmol/l E_2, 0.1 μmol/l Gen and 0.1 μmol/l 8-PN. In a pilot experiment, it appeared that dilution of sperm suspensions into medium without the test compound just before addition of oocytes had no detectable effect on E_2-treated suspensions, but it did appear to have an effect on 8-PN-treated suspensions. Therefore, in subsequent experiments ($n = 3$), suspensions treated with 8-PN and Gen were diluted into medium with and without the test compound in order to determine whether a reduction in their concentration had any detectable effect on sperm function; this resulted in there being more oocytes in the 8-PN and Gen test groups than in the controls and E_2-treated groups.

Table I. 17β-Estradiol (E_2), genistein (Gen) and 8-prenylnaringenin (8-PN) significantly stimulate fertilizing ability in uncapacitated mouse sperm suspensions

Treatment	Oocytes fertilized/ total oocytes	Range (%)
None (control)	45/127 (35.4)	23–53
10 μmol/l E_2	104/136 (76.5)***	68–83
0.1 μmol/l Gen	178/224 (79.5)***	65–83
0.1 μmol/l 8-PN	197/268 (73.5)***	70–80

$^{***}P < 0.01$ compared with untreated controls ($n = 3$)
Values in parentheses are percentages.

All three compounds significantly ($P < 0.01$) stimulated fertilizing ability, compared with untreated controls (Table I). The fertilization rate (proportion of oocytes penetrated) was similar whether sperm suspensions were diluted into medium with or without test compound, and so only combined data are shown. However, although there were no obvious differences in the two treatments with Gen, with 8-PN it was consistently observed that sperm penetration was faster when the 8-PN concentration was maintained at 0.1 μmol/l. In that group, there were more fully decondensed sperm heads within oocytes than in the group where suspensions were simply diluted into normal culture medium; in the latter, the sperm heads were at various earlier stages of decondensation. This may indicate that hyperactivated motility is supported more effectively by the presence of the higher concentration of 8-PN.

To ensure that there were enough oocytes in each replicate to obtain meaningful data, it was decided to test E_2 and two of the three environmental estrogens. Consequently NP was not tested, but since CTC results with NP were similar to those seen with the other environmental estrogens, enhanced fertilizing ability of NP-treated suspensions would be predicted.

Discussion

The present study addressed the question of whether naturally occurring estrogens and environmental estrogenic compounds have any effect on mammalian sperm function. Uncapacitated epididymal mouse sperm were treated with increasing concentrations of E_2 and the environmental estrogens Gen, 8-PN and NP for 30 min, then assessed using CTC analysis. E_2 used at 1–100 μmol/l and Gen, 8-PN and NP used at 0.001–1 μmol/l significantly stimulated capacitation and the acrosome reaction, compared with untreated controls (Figures 1–4). Thus, although all compounds had an effect, the environmental estrogens were considerably more potent than E_2. In capacitated sperm, E_2 used at 0.01–10 μmol/l had no significant effect (Figure 5), but Gen, 8-PN and NP all significantly stimulated the acrosome reaction, compared with untreated controls (Figure 6). Of the environmental estrogens, Gen and 8-PN were more potent, having maximal effect at 0.01 μmol/l and above, while NP was maximally effective at 1 μmol/l. In

addition to CTC analysis, which is an indirect method, assessment of treated sperm using IVF confirmed and extended the CTC results, namely that E_2 at 10 μmol/l and Gen and 8-PN, each at 0.1 μmol/l, significantly stimulated capacitation and demonstrable fertilizing ability (Table I).

The results obtained in this study contrast with those published in some recent studies. Others (Luconi *et al*., 1999, 2001) reported that E_2 inhibited the progesterone-induced acrosome reaction in human sperm, but that the environmental estrogens bisphenol A and octylphenol polyethoxylate had no detectable effect. It was also reported (Hinsch *et al*., 2000) that genistein significantly inhibited the progesterone-induced acrosome reaction in cryopreserved bull sperm, but the concentration used (2 μg/ml, ~7.4 μmol/l) was considerably higher than the highest concentration used in the present study (1 μmol/l). Therefore, the present study has provided the first evidence that E_2 and environmental estrogens can significantly stimulate sperm function. Interestingly, E_2 was able to elicit significant responses in uncapacitated but not in capacitated cells, while the environmental estrogens were able to act on both categories of sperm.

Gen, 8-PN and NP are able to bind to both estrogen receptors, ERα and ERβ, but with lower affinity than E_2 (Kuiper *et al*., 1998; Milligan *et al*., 1999, 2000). The estrogenic activity of these compounds is also considerably less than that of E_2 when assessed by bioassays. For example, in an in-vitro yeast screen employing the human ERα, the relative potencies of 8-PN, Gen and NP were <1/100, 1/1000 and <1/10 000 respectively that of E_2 (Routledge and Sumpter, 1996; Milligan *et al*., 1999). Since the environmental estrogens in the present study were very effective at low (nanomolar) concentrations, the results obtained in this study would appear to be positive physiological responses rather than negative, toxic responses. Much higher concentrations have been used on other cell types with no apparent ill effects, either *in vitro* (above) or *in vivo*. For example, one group (Kwack *et al*., 2002) obtained a positive in-vivo uterotrophic response to NP, but needed 200–400 mg/kg per day NP compared with only 1 μg/kg per day E_2.

The fact that the environmental estrogens proved to be markedly more potent than E_2 in stimulating sperm function was surprising, given their generally low potency in estrogenic bioassays (above). The inability of hydroxytamoxifen to inhibit responses to either E_2 or the environmental estrogens (Figure 7) suggests that the responses observed in the present study do not involve classic ERs. Nanomolar concentrations of Gen, 8-PN and NP stimulated capacitation in uncapacitated cells, compared with the micromolar concentrations required for E_2, raising the possibility that E_2 and the environmental compounds might be binding either to different receptors or to a non-classical ER with different properties from ERα and β. The rapidity of response seen in transcriptionally inactive sperm suggests that it is likely that E_2 and the other compounds are acting via a non-genomic route (Luconi *et al*., 2002), perhaps by binding to some surface membrane receptor or entering the cell and then binding to an intracellular receptor. The presence of an intracellular receptor for E_2, distinct from the one used by Gen, 8-PN and NP, could explain how all four had a significant effect on uncapacitated sperm, yet E_2 had no effect on capacitated cells. During capacitation, the composition and structural arrangement of the sperm plasma membrane components change (de Lamirande *et al*., 1997), perhaps rendering the E_2 binding site inaccessible.

At present there is no conclusive evidence as to how either E_2 or environmental estrogens might exert non-genomic effects in sperm. Investigation of ERα and ERβ expression in the mouse testis during fetal and post-natal development detected mRNA for both ERs in the fetal testis. ERα protein was localized primarily in interstitial regions, while ERβ protein was detected in germ cells from day 16 of gestation; by 26 days post partum, no ERβ protein could be detected (Jefferson *et al*., 2000). Although these data suggest that mouse sperm do not have ERs, other studies have reported immunocytochemical and other evidence for the presence of ERs in both human (Misao *et al*., 1997; Durkee *et al*., 1998) and rat sperm (Saberwal *et al*., 2002). A surface membrane protein has been partially purified and characterized as the putative non-genomic receptor for E_2 in human sperm (Luconi *et al*., 1999), but this remains to be substantiated, especially as the size (~29 kDa) is considerably smaller than that of ERα and ERβ. Using Western blotting, one group (Durkee *et al*., 1998) reported detecting a single band of ~65 kDa (appropriate size for ER) in human sperm, while very recently an ERα of ~66 kDa was reported in rat sperm (Saberwal *et al*., 2002).

In addition to uncertainty about the molecules with which E_2 and other estrogenic compounds initially interact, there appear to be a number of different mechanisms/pathways that could be involved in the responses. Even if the estrogenic compounds evaluated in the present study are acting via receptors that differ from the classic ERs, it is possible that the responses may involve the same signal transduction pathway(s). In various somatic cell systems, E_2 has been reported to modulate Ca^{2+} fluxes, generate cyclic nucleotides, activate various kinases, and modulate ion channels (Kelly and Levin, 2001). Recent studies have shown that the adenylyl cyclase (AC)/cAMP pathway is very important in mammalian sperm, with a rise in cAMP significantly stimulating capacitation (Fraser and Adeoya-Osiguwa, 2001). In human sperm, it has been reported that E_2 initiated rapid increases in both intracellular Ca^{2+} ($[Ca^{2+}]_i$; the response being biphasic in the presence of 10 μmol/l E_2) and protein tyrosine phosphorylation (Luconi *et al*., 1999, 2001). Such a rise in $[Ca^{2+}]_i$ might stimulate the AC/cAMP signal transduction pathway, resulting in increased tyrosine phosphorylation (Visconti *et al*.., 1995; Adeoya-Osiguwa and Fraser, 2000). In a recent study, male rats were given 400 μg/kg per day of tamoxifen citrate for 60 days, and various sperm parameters were then evaluated. Calcium and cAMP levels were significantly higher in sperm from treated rats than in those from untreated controls; unfortunately, no evaluation of their fertility was undertaken, but the authors suggested that these changes resulted from tamoxifen binding to ERs and acting as an agonist (Saberwal *et al*., 2002).

In contrast to the above positive responses to E_2, genistein at fairly high concentrations (≥2 μmol/l) was reported to inhibit tyrosine phosphorylation of several proteins thought to play a role in capacitation (Carrera *et al*., 1996). In the present study,

both E_2 and genistein had a similar, stimulatory effect on uncapacitated cells and capacitation is associated with an increase, not a decrease, in protein phosphorylation (Visconti *et al*., 1995; Adeoya-Osiguwa and Fraser, 2000). This makes it unlikely that the observed stimulation of sperm function involves inhibition of tyrosine phosphorylation, but a mechanism that involved stimulation of AC/cAMP is plausible.

The regulation of capacitation is very important in mammalian fertilization as evidence now suggests that once capacitation has been initiated it will usually continue unchecked, frequently resulting in sperm undergoing spontaneous acrosome reactions and thus becoming non-fertilizing (Fraser and Adeoya-Osiguwa, 2001). Recent in-vitro studies have indicated that a number of peptides, found in seminal plasma and known to have specific receptors on mammalian sperm, can regulate capacitation, initially stimulating capacitation and then inhibiting spontaneous acrosome loss (Fraser and Adeoya-Osiguwa, 2001). Responses to fertilization promoting peptide (FPP), adenosine and calcitonin all involve G protein-mediated regulation of AC/cAMP, initially stimulating the production of cAMP and then inhibiting it. Because sperm always contact these molecules *in vivo* at the time of ejaculation, it has been proposed that they may provide an important mechanism for maximizing the number of potentially fertilizing capacitated, acrosome-intact sperm available within the female reproductive tract.

In contrast to those regulated responses, E_2 and the environmental estrogens appear primarily to stimulate sperm, accelerating the rate of capacitation and then promoting 'over-capacitation' in at least some of the cells, resulting in the acrosome reaction. Since already acrosome-reacted sperm are non-fertilizing (Yanagimachi, 1994), similar responses occurring *in vivo* could reduce the number of potentially fertilizing cells and so have an undesirable effect on fertility. On the other hand, it is possible that sperm exposed to both the seminal plasma peptides and various estrogenic compounds may have the best of both worlds, with the initial stimulatory responses to all molecules subsequently being regulated by FPP and the others, thus ensuring that most of the sperm retain fertilizing potential. As capacitation and fertilization occur in the female reproductive tract, it is likely that any effects of environmental estrogens on sperm function would be more pronounced in the female, but effects on mature sperm awaiting ejaculation cannot be ruled out.

In conclusion, this study has provided the first direct evidence that E_2 and environmental estrogens significantly affect the function of mature sperm by stimulating capacitation and fertilizing ability, compared with untreated controls. Given the much greater potency of environmental estrogens than E_2 on sperm, and the likelihood that many individuals will be exposed to more than one of these compounds at any given time (albeit at low concentrations of each), these results clearly indicate that further investigation is very important. The implications that these results may have for in-vivo fertility in humans and domestic animals cannot be deduced until the mechanism(s) by which these compounds act are identified and fully understood, making it vitally important to extend these investigations.

References

Adeoya-Osiguwa, S.A. and Fraser, L.R. (2000) Fertilization promoting peptide and adenosine, acting as first messengers, regulate cAMP production and consequent protein tyrosine phosphorylation in a capacitation-dependent manner. *Mol. Reprod. Dev*., **57**, 384–392.

Adeoya-Osiguwa, S.A., Markoulaki, S., Pocock, V., Milligan, S.R. and Fraser, L.R. (2002) Effects of estradiol 17β and environmental oestrogens on mammalian sperm function. *Hum. Reprod.*, **17** (Abstract Bk. I), p. 42.

Akingbemi, B.T. and Hardy, M.P. (2001) Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. *Ann. Med.*, **33**, 391–403.

Anstead, G.M., Carlson, K.E. and Katzenellenbogen, J.A. (1997) The estradiol pharmacophore: ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids*, **62**, 268–303.

Aquila, S., Sisci, D., Gentile, M., Middea, E., Siciliano, L. and Ando, S. (2002) Human ejaculated spermatozoa contain active P450 aromatase. *J. Clin. Endocrinol. Metab.*, **87**, 3385–3390.

Ashby, J. (2001) Testing for endocrine disruption post-EDSTAC: extrapolation of low dose rodent effects to humans. *Toxicol. Lett*., **120**, 233–242.

Austin, C.R. (1951) Observations on the penetration of the sperm into the mammalian egg. *Aust. J. Sci. Res*., B4, 581–596.

Austin, C.R. (1952) The ''capacitation'' of the mammalian sperm. *Nature*, **170**, 326.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R. and Sheehan, D.M. (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci*., **54**, 138–153.

Carreau, S., Genissel, C., Bilinska, B. and Levallet, J. (1999) Sources of oestrogen in the testis and reproductive tract of the male. *Int. J. Androl.*, **22**, 211–223.

Carrera, A., Moos, J., Ning, X.-P., Gerton, G.L., Tesarik, J. and Kopf, G.S. (1996) Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. *Dev. Biol.*, **180**, 284–296.

Cassidy, A. and Milligan, S.R. (1998) How significant are environmental estrogens to women? *Climacteric*, **1**, 229–242.

Chang, M.C. (1951) Fertilizing capacity of spermatozoa deposited into Fallopian tubes. *Nature*, **168**, 697–698.

Cheng, C.Y., Boettcher, B. and Rose, R.J. (1981a) Lack of cytosol and nuclear estrogen receptors in human spermatozoa. *Biochem. Biophys. Res. Commun.*, **100**, 840–846.

Cheng, C.Y., Boettcher, B., Rose, R.J., Kay, D.J. and Tinneberg, H.R. (1981b) The binding of sex steroids to human spermatozoa. An autoradiographic study. *Int. J. Androl.*, **3**, 23–29.

de Lamirande, E., Leclerc, P. and Gagnon, C. (1997) Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.*, **3**, 175–194.

Durkee, T.J., Mueller, M. and Zinaman, M. (1998) Identification of estrogen receptor protein and messenger ribonucleic acid in human spermatozoa. *Am. J. Obstet. Gynecol.*, **178**, 1288–1297.

Fraser, L.R. (1984) Mouse sperm capacitation *in vitro* involves loss of a surface-associated inhibitory component. *J. Reprod. Fertil.*, **72**, 373–384.

Fraser, L.R. (1993) *In vitro* capacitation and fertilization. In Wasserman, P.M. and DePamphilis, M.L. (eds), *Guide to Techniques in Mouse Development*. *Methods Enzymol.*, **225**, 239–253.

Fraser, L.R. and Adeoya-Osiguwa, S.A. (2001) Fertilization promoting peptide – A possible regulator of sperm function in vivo. *Vit. Horm.*, **63**, 1–28.

Green, C.M., Cockle, S.M., Watson, P.F. and Fraser, L.R. (1994) Stimulating effect of pyroglutamylglutamylprolinamide, a prostatic, TRH-related tripeptide, on mouse sperm capacitation and fertilizing ability in vivo. *Mol. Reprod. Dev.*, **45**, 244–252.

Green, C.M., Cockle, S.M., Watson, P.F. and Fraser, L.R. (1996) Fertilization promoting peptide, a tripeptide similar to thyrotrophin-stimulating hormone, stimulates the capacitation and fertilizing ability of human spermatozoa *in vitro*. *Hum. Reprod.*, **11**, 830–836.

Hernandez-Perez, O., Ballesteros, L.M. and Rosado, A. (1979) Binding of 17-β estradiol to the outer surface and nucleus of human spermatozoa. *Arch. Androl.*, **4**, 1–17.

Hess, R.A. (2000) Oestrogen in fluid transport in efferent ducts of the male reproductive tract. *Rev. Reprod.*, **5**, 84–92.

Hess, R.A., Bunick, D., Lee, K.-H. Bahr, J, Taylor, J.A., Korach, K.S. and Lubahn, D.B. (1997) A role for oestrogens in the male reproductive system. *Nature*, **390**, 509–511.

Hinsch, K.D., Aires, V., Hagele, W. and Hinsch, E. (2000) *In vitro* tests for essential sperm functions using the phyto-oestrogen genistein as a test substance. *Andrologia*, **32**, 225–231.

Jefferson, W.N., Couse, J.F., Banks, E.P., Korach, K.S. and Newbold, R.R. (2000) Expression of estrogen receptor beta is developmentally regulated in reproductive tissues of male and female mice. *Biol. Reprod.*, **62**, 310–317.

Kelly, M.J. and Levin, E.R. (2001) Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol. Metab.*, **12**, 152–156.

Korach, K.S., Couse, J.F., Curtis, S.W., Washburn, T.F., Lindzey, J., Kimbro, K.S., Eddy, E.M., Migliaccio, S., Snedeker, S.M., Lubahn, D.B. *et al.* (1996) Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog. Horm. Res.*, **51**, 59–186.

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J.A. (1998) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, **138**, 863–870.

Kwack, S.J., Kwon, O., Kim, H.S., Kim, S.S., Kim, S.H., Sohn, K.H., Lee, R.D., Park, C.H., Jeung, E.B., An, B.S. *et al.* (2002) Comparative evaluation of alkylphenolic compounds on estrogenic activity *in vitro* and in vivo. *J. Toxicol. Environ. Health*, **65**, 419–431.

Levin, E.R. (2001) Cell localization, physiology, and nongenomic actions of estrogen receptors. *J. Appl. Physiol.*, **91**, 1860–1867.

Luconi, M., Muratori M., Forti, G. and Baldi, E. (1999) Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J. Clin. Endocrinol. Metab.*, **84**, 1670–1678.

Luconi, M., Bonaccorsi, M., Forti, G. and Baldi, E. (2001) Effects of estrogenic compounds on human spermatozoa: evidence for interaction with a nongenomic receptor for estrogen on human sperm membrane. *Mol. Cell. Endocrinol.*, **178**, 39–45.

Luconi, M., Forti, F. and Baldi, E. (2002) Genomic and nongenomic effects of estrogens: molecular mechanisms of action and clinical implications for male reproduction. *J. Steroid Biochem. Mol. Biol.*, **80**, 369–381.

Mazur, W. and Adlercreutz, H. (2000) Overview of naturally occurring endocrine-active substances in the human diet in relation to human health. *Nutrition*, **16**, 654–658.

Milligan, S.R., Kalita, J.C., Heyerick, A., Rong, H., De Cooman, L. and De Keukeleire, D. (1999) Identification of a potent phytoestrogen in hops (*Humulus lupulus* L.) and beer. *J. Clin. Endocrinol. Metab.*, **83**, 2249–2252.

Milligan, S.R., Kalita, J.C., Pocock, V., Van de Kauter, V., Stevens, J.F., Deinzer, M.L., Rong, H., and De Keukeleire, D. (2000) The endocrine activities of 8-prenylnaringenin and related hop (*Humulus lupulus* l.) flavonoids. *J. Clin. Endocrinol. Metab.*, **85**, 4912–4915.

Milligan, S.R., Kalita, J.C., Pocock, V., Heyerick, A., De Cooman, L., Rong, H. and De Keukeleire, D. (2002) Oestrogenic activity of the hop phyto-estrogen, 8-prenylnaringenin. *Reproduction*, **123**, 235–242.

Misao, R., Niwa, K., Morishita, S., Fujimoto, J., Nakanishi, Y. and Tamaya, T. (1997) Immunohistochemical detection of estrogen and progesterone receptors in spermatozoa of infertile men. *Int. J. Fertil.*, **42**, 421–425.

Revelli, A., Massobrio, M. and Tesarik, J. (1998) Nongenomic actions of steroid hormones in reproductive tissues. *Endocr. Rev.*, **19**, 3–17.

Robertson, K.M., O'Donnell, L., Jones, M.E., Meachem, S.J., Boon, W.C., Fisher, C.R., Graves, K.H., McLachlan, R.I. and Simpson, E.R. (1999) Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc. Natl Acad. Sci. USA*, **96**, 7986–7991.

Routledge, E.J. and Sumpter, J.P. (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.*, **15**, 241–258.

Saberwal, G.S., Sharma, M.K., Balasinor, N., Choudhary, J. and Juneja, H.S. (2002) Estrogen receptor, calcium mobilization and rat sperm motility. *Mol. Cell. Biochem.*, **237**, 11–20.

Sharpe, R.M. (2001) Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol. Lett.*, **120**, 221–232.

Skakkebaek, N.E., Rajpert-De Meyts, E. and Main, K.M. (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum. Reprod.*, **16**, 972–978.

Snedecor, G.W. and Cochran, W.G. (1980) *Statistical Methods*. 7th edn, Iowa State University Press, Ames, Iowa, USA.

Sohoni, P. and Sumpter, P.J. (1998) Several environmental estrogens are also anti-androgens. *J. Endocrinol.*, **158**, 327–339.

Sonnenschein, C. and Soto, A. (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J. Steroid Biochem. Mol. Biol.*, **65**, 143–150.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P. and Kopf, G.S. (1995) Capacitation of mouse spermatozoa. II: protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*, **121**, 1139–1150.

Yanagimachi, R. (1994) Mammalian fertilization. In Knobil, E. and Neill, J.D. (eds), *The Physiology of Reproduction.* 2nd edition. Raven Press, New York, pp. 189–317.

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