Effect of estradiol on expression of cytoskeletal proteins during spermatogenesis in testis of sexually mature rats


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Received 7 February 2005

Earlier studies had shown that long term treatment with estradiol arrests spermatogenesis in adult male rats, at a dose of 0.1 mg/kg/day. The present study was therefore undertaken to ascertain the causes underlying the reduction in sperm counts by administering estradiol for a short term, at the dose of 0.1 mg/kg/day, for a period of 10 days to one group of adult male rats, which were administered saline for 12 days prior to estradiol injection, and sacrificed after 22 days. The control group was administered saline for 22 days. The sera were analyzed for testosterone and FSH levels. One testis of each male was immersion fixed for histology, and for immunohistochemistry of two testicular cytoskeletal proteins, vimentin and vinculin. The contralateral testes were used for analysis of vimentin and vinculin gene expression by reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting. Another group exposed to estradiol for 10 days was injected with bromodeoxyuridine (BrdU), at a dose of 100 mg/kg/day, to ascertain the effect on germ cell proliferation, and sacrificed 12 days later, while estradiol treatment was continued till sacrifice. BrdU, at a dose of 100 mg/kg/day was injected i.p. to a group of control rats treated with saline for 10 days, and sacrificed 12 days later. The testes from both groups were immersion fixed for immunohistochemical detection of BrdU. Histology of estradiol treated testis showed predominance of tubules with round spermatids with accumulation of lipid droplets in Sertoli cell cytoplasm and decreased cell height, whereas controls showed elongating spermatids. BrdU immunolocalization in the testis, irrespective of treatment, indicated its incorporation in deoxyribonucleic acid (DNA) suggesting that estradiol sustained germ cell proliferation. Both vimentin and vinculin could be immunolocalized to the testis. The testicular levels of vimentin and vinculin, quantified after western blotting, were unaffected. The testicular expression of vimentin and vinculin seen by RT-PCR was also unaffected. The study suggested that estradiol induced reduction in sperm counts was not due to adverse effects on proliferation. The observed predominance of seminiferous tubules showing round spermatids, accumulation of lipid droplets as compared to controls suggested that reduction in elongated spermatids occurred through reduced spermatogenesis and phagocytosis. The study also suggested that reduction in Sertoli cell height after short-term estradiol treatment was not due to reduced expression of vimentin and vinculin, which could be maintained by estradiol. However, reduction in Sertoli cell height could have been due to suppression of FSH and testosterone, implicated in the polymerization of vimentin and organization of vinculin, two cytoskeletal proteins involved in inter-Sertoli or Sertoli-germ cell junctions. The study suggested that disorganization of Sertoli cell cytoskeleton and reduction in the volume of Sertoli cells could be an important factor for reduced efficiency of spermatogenesis after exposure to estrogenic molecules.

Keywords: Estradiol, Sertoli cell morphology, Sperm counts, Vimentin, Vinculin

In recent years, significant advances have been made in the field of cell to cell interactions within the testis, not only in identifying the types of junctions between the somatic and germ cells but also the proteins involved in their formation within the seminiferous tubules. Several types of anchoring junctions namely tight junctions, adherens junctions, desmosomes, hemidesmosomes have been described through which Sertoli cells communicate with each other, germ cells or matrix proteins, create the blood testis barrier and divide the seminiferous tubules into the basal and adluminal compartments. Several proteins integral to the different types of junctions have been identified. The junctional proteins...
Observations made over the years had indicated that evidence suggested that prolonged FSH deprivation organization of cytoskeleton of the ectoplasmic specializations and preventing the attachment of spermatocytes and spermatogonia in adult rat \cite{5}. The available evidence suggested that prolonged FSH deprivation primarily reduces sperm counts by interfering with the organization of cytoskeleton of the ectoplasmic specializations and preventing the attachment of round spermatids \cite{14}. The effects of FSH in inducing morphological changes in Sertoli cells in vitro concomitant with phosphorylation of vimentin intermediate filaments is well documented \cite{16}. Expression of cytoskeletal protein vimentin is characteristic of mature Sertoli cell wherein it is upregulated during stages XII-V concomitant with the expression of FSHR in stages XII-II of spermatogenesis in rats \cite{17,18}. Vimentin has been implicated in steroidogenesis in Leydig cells but an identical role has not been reported in Sertoli cells \cite{19}. Morphological and functional changes in Sertoli cells are reported to occur during spermatogenesis, where increase in lipid droplets occurs, (following a peak of primary lysosomes in stage VIII for degradation of residual bodies phagocytosed by Sertoli cells after spermiogenesis), in stages IX to II (coinciding with a peak of cathepsin A positive secondary lysosomes formed after digestion of residual bodies) with a peak in stage XII (representing the fusion of secondary lysosomes with phagocytosed late residual bodies) \cite{2,20}.

Although the mechanism underlying the induction of proliferation of germ cells remains elusive as the germ cells do not express FSH receptors, FSH is involved in the aromatization of testosterone to estradiol in Sertoli cells \cite{21}. The efficiency of aromatization is very high in immature Sertoli cells and progressively reduces at adulthood \cite{22}. Recently, estradiol has been shown to induce Sertolin in Sertoli cells, a putative negative regulator of proteases involved in formation of Sertoli germ cell junctions. Estradiol has the potential to suppress the formation of junctions between Sertoli and germ cells \cite{23}. An effect on Sertolin expression could possibly be a valid reason underlying the failure of deletion of estrogen receptor $\beta$ to render estrogen receptor $\beta$ knockout mice (BERKO) infertile \cite{24}. Although the precise role of estradiol during spermatogenesis is not known, in vitro and in vivo studies have shown it induces germ cell proliferation and survival \cite{25}. Estradiol implants have been reported to restore spermatogenesis in hypogonadal hpg mice \cite{26}. Estrogen receptor $\beta$ is reported to be upregulated in the highly proliferative rat gonocytes \cite{27}. Within the seminiferous tubules, estrogen receptor $\beta$, co-expressed with cytochrome $P_{450}$ aromatase in the Sertoli and germ cells, is transiently downregulated in spermatogonial stem cells followed by increase in expression in subsequent lineages \cite{27,28,29}. Estrogen receptor $\beta$ may be involved in acrosome biogenesis in differentiating spermatids wherein cytochrome $P_{450}$ aromatase is also co-expressed in the Golgi apparatus \cite{32}. Estrogen receptor $\alpha$
has been implicated in the resorption of fluid and sperm maturation in different ductules. Thus, FSH and estradiol have the potential to sustain germ cell proliferation in a coordinated, cell-specific manner, through expression of genes involved in germ cell proliferation as well as organization of cytoskeletal proteins involved in the regulation of Sertoli cell junctions and volume. Any effects on the expression of cytoskeletal protein genes involved in the regulation of Sertoli cell volume could have crucial implications for germ cell binding potency, permitting differentiation of a maximum number of germ cells and maintenance of sperm counts characteristic of a given species.

Testosterone, the principal reproductive hormone involved in the differentiation of stage VII round spermatids, is the substrate for aromatization in the Sertoli cells. Chowdhury and Steinberger first observed that estrogen antagonizes the androgen-dependent differentiation of immature rodent Sertoli cells. Thus, down regulation of aromatization with a shift to androgen regulation at adulthood, could well be integral to Sertoli cell differentiation. Studies with mutant hpg mice revealed that testosterone restored cytodifferentiation of round spermatids and sperm counts. Muffly et al also observed that androgen can maintain the adhesion of germ cells if given immediately after hypophysectomy in rats. O’Donnell et al first reported that androgen is essential for adhesion of stage VII round spermatids and their differentiation to elongated spermatids in rats bearing TE (testosterone plus estradiol) implants. Spermiation failure observed in the same TE rat model after treatment with FSH antibody had led the authors to study the effect on proteins of ectoplasmic specializations (ES). These studies led to the conclusion that regulatory effects of androgens and FSH on cell adhesion proteins of non-ES type of anchoring junctions were involved in spermiation failure. A failure of the androgen-dependent adhesion mechanism(s) affects sperm counts presumably due to sloughing of stage VII round spermatids and to a certain extent, abnormal retention of mature stage VIII spermatocytes at the time of spermiation. Subsequent studies by O’Donnell et al. revealed that the intensity of immunorexpression of cytoskeletal proteins associated with the ectoplasmic specializations involved in recruiting, attachment and positioning of spermatids in the crypts of the Sertoli cells appeared to be affected in rats bearing TE implants. At adulthood, androgen receptors, expressed selectively in Sertoli cell, are upregulated in spermatogenic stages VII-XII in rat, whereas L-tyg hydroptrophy indicative of increased testosterone synthesis occurs in stages VII-VIII. Sertoli cell differentiation reportedly involves the expression of vimentin and disappearance of cytokeratin, apart from downregulation of aromatization. Recently, reduction in intratesticular androgen by TE implants has been reported to reduce the levels of vimentin monomers in rat testis, implicated in cell to cell interactions through vimentin based junctions, due to degradation.

Estrogen treatment is known to suppress both FSH and testosterone. The estrogen receptor beta has been reported to increase in at least two models of androgen deficiency, LH receptor knockout (LHRKO) and androgen binding protein (ABP) transgenic mice. Presumably, the basis of antagonism between the two steroidal hormones would be the modulation of putative androgen dependent genes by estrogen receptor beta. Interactions between FSH and the two steroids could thus be of physiological relevance in the stage or cell specific expression and organization of cytoskeletal proteins in the Sertoli cells and maintenance of species-specific sperm counts. Failure of spermiation after treatment with antibody to FSH already reported in rats bearing TE implants and the selective apoptotic loss of round spermatids, a known characteristic of withdrawal of testosterone in rats treated with ethanedimethanesulphonate (EDS), could well be related to breakdown of vimentin filaments in the testis of these rats. The present study is an attempt to correlate the expression of vimentin and vinculin, implicated in maintaining Sertoli cell anchoring junctions, to reduced efficiency of spermatogenesis in adult rats exposed to estradiol, with a view to gain insights into the molecular basis of its neuroendocrine effects.

**Materials and Methods**

**Animals**—Sexually mature 75 day old male rats of the Holtzman strain, bred at NIRRH (National Institute of Research and Reproductive Health), Mumbai, were maintained at a temperature of 22°-23°C, 50-55% humidity and light/dark cycle of 14:10 hr. Commercial food pellets and water were available ad libitum. Control and experimental rats were divided into four groups of 6 rats each. All
experiments were carried out with the permission of the ethical committee of NIRRH.

Drugs—Estradiol valerate (Sigma, USA) was uniformly suspended in saline by sonication and administered subcutaneously to male rats, at a dose of 0.1 mg/kg body weight, between 10:00-12.00 hr for 10-20 days so as not to cause arrest of spermatogenesis. The dose of estradiol was selected on the basis of an earlier study in which it was found to be effective in suppressing sperm counts within 60 days. A 2% aqueous solution of 5-Bromodeoxyuridine (BrdU, Sigma, USA) was prepared and administered intraperitoneally at a dose of 100 mg/kg body weight for 12 days.

Treatment schedule—The total period of experiment was 22 days. The control group of rats was administered saline and sacrificed 22 days later. The experimental group of rats kept on saline for 12 days, was sacrificed after estradiol administration for a further period of 10 days. A control group of rats (for histology/immunohistochemistry) kept on saline for 10 days, was sacrificed after BrdU injection for a further period of 12 days. The experimental group of rats treated with estradiol for 10 days, was injected with BrdU and sacrificed 12 days later, while estradiol treatment was continued till sacrifice (to avoid reversal of effect).

Autopsy of male rats—Male rats were killed by decapitation. Blood from the trunk region was collected and allowed to clot at 4°C. The analysis of serum FSH, LH, testosterone, and estradiol was done by radioimmunoassays. One testis from each rat was immersion fixed in modified Karnovsky’s fixative for routine histology in semi thin araldite sections and in Bouin’s fixative for immunohistochemistry. The contralateral testis from each rat was processed for vimentin and vinculin RNA extraction for RTPCR.

Histology—Histology was performed as described earlier and evaluation of spermatogenesis done in semi thin sections of 0.5 um thickness. Testicular tissues from each group of rats were fixed in modified Karnovsky’s fixative, washed in 0.1M sodium cacodylate buffer, post fixed in 1% osmium tetroxide, dehydrated in ascending series of acetone (30-100%), and embedded in Araldite. Tissues were stained with 1% toluidine blue and observed under bright-field optics at 40/100 X magnifications. Sertoli cell height was determined in 10 testicular sections from all rats with an ocular micrometer under a microscope and average height of 10 sections from 6 rats was expressed as mean height in um±SD.

Hormone assays—LH, FSH, testosterone and estradiol were assayed as described earlier. The standard curves for LH (NIADDK-rat LH-RP-2) and FSH (NIADDK-rat FSH-RP-2) ranged from 10 pg to 12.5 ng per assay tube. The inter and intra assay coefficients of variation were 9% and 6% for LH, 10 and 6% for FSH, respectively. The standard curves for testosterone and estradiol ranged from 3.9 to 500 pg. Inter and intra assay coefficients of variation were 5.5 and 11% for testosterone, and 6 and 10% for estradiol, respectively.

Immunolocalization of vimentin and vinculin in testis—The testes were fixed in Bouin’s fixative for 48 hr. The tissues were dehydrated through 30, 50, 70, 80, 90, 95, and 100% alcohol grades for 1 hr each with 2 changes, followed by 2 washes of 0.5 hr each in xylol (1:1 alcohol : xylene) and cleared with xylene for 2 hr. Tissues were then kept in wax at 56°C overnight prior to making blocks. Sections of 5um thickness were cut and taken onto slides coated with poly-L-lysine (1:10 dilution). The slides were dewaxed in xylene for 10 min and further cleared in fresh xylene for 10 min. Slides were blotted and immersed in 100% methanol for 15 min. The slides were kept in hydrogen peroxide (H2O2) prepared in 100% methanol (1ml of 30% H2O2 in 49 ml of methanol) for 45 min and washed with distilled water (DW) for 5 min followed by two washes in phosphate buffered saline (PBS) (0.01M pH 7.4) for 5 min each.

The slides were then blocked with 1% normal donkey serum (NDS) in PBS for 1 hr at room temperature (RT). The slides were then incubated overnight with primary vimentin or vinculin antibodies at 1:25 dilution in 1% normal donkey serum (NDS, Santacruz Biotechnology, USA) at 4°C. The negative controls were incubated with normal goat serum (NGS) (50µl in 1 ml PBS 0.01M; pH 7.4). The slides were washed in PBS 0.01M pH 7.4, thrice for 10 min each. The slides were incubated for 2 hr with secondary antibody (donkey antigoat, Santacruz Biotechnology, USA) at 1:100 dilution in 1% NDS diluted in PBS at RT. The slides were washed in PBS thrice for 10 min each. The slides were incubated with avidin biotin complex (ABC) in PBS for 45 min and washed in PBS thrice for 10 min each. The slides were then

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developed with diaminobenzoic acid (DAB) (5mg/10ml PBS 0.01 M containing 15 μl of 30% hydrogen peroxide) for 5 min, washed with distilled water (DW) and counterstained with aqueous haematoxylin for 10 min. The slides were washed with DW, dipped in 100% alcohol, air dried, and put in xylene overnight prior to mounting in DPX and viewed under bright field microscope at 40x.

**Immunolocalization of BrdU in testis**—BrdU was localized in the nuclei of testicular cells with anti-BrdU antibody (Sigma, USA). Paraffin sections of testis (5μm) were dewaxed by warming on a hot plate. The slides were cleared in xylene for 10 min. The sections were then rehydrated by dipping once in 100, 90, 70, 50, 30% alcohol, DW, and twice in PBS (pH 7.4) for 10 min each. Endogenous peroxidase activity in the tissues was quenched by incubating the sections in 2% hydrogen peroxide diluted in PBS for 15 min. The slides were washed once with distilled water and PBS. The slides were covered with aqueous 0.01 calcium chloride (pH 7.8) containing 0.1% trypsin and kept at 37°C for 20 min in a porcelain jar followed by 2 N HCl for 1hr at 37°C. The slides were blocked with 1% bovine serum albumin (BSA) for 1 hr at RT followed by incubation with primary monoclonal antibody (at a dilution of 1:500 in 0.01% PBS 0.01 M) overnight at 4°C. The slides were washed thrice for 5 min each in PBS followed by incubation with biotinylated secondary antibody (horse anti-mouse) at a dilution of 1:500 for 1 hr at RT, followed by three washes of 10 min each in PBS 0.01 M. The slides were then incubated with horsradish peroxidase (HRP) conjugated avidin-biotin complex (Vectastain, USA) for 1hr followed by two washes of 10 min each. The BrdU was visualized with DAB (5mg/10ml of 0.01M PBS containing 15 μl of 30% hydrogen peroxide) following which the reaction was terminated by immersing the slides in DW. Some sections were not counterstained, while few were counterstained with 5% aqueous solution of eosin in water for 1 min. The slides were dipped once in 70, 95%, and absolute alcohol, air dried and left in xylene over night and then mounted in DPX. The primary antibody was substituted by PBS (0.01 M) in negative controls. The sections were photographed under bright field optics at 40x magnification.

**Detection of vimentin and vinculin by western blotting in testis**—Rat testicular tissues (50 mg) were homogenized in 1 ml of RIPA buffer (50 mM Tris base pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS and protease inhibitors) at maximum speed in a mechanized homogenizer. The homogenates were centrifuged at 15,000 rpm for 30 min at 4°C. The supernatants containing the cytoskeletal proteins were stored at -20°C. The proteins in the homogenates were estimated by the method of Lowry.

Aliquots of testicular homogenates containing 100 μg of proteins were mixed with an equal volume of 2x sample buffer, heated at 95°C for 5 min, cooled on ice and loaded in wells. Molecular weight markers in the range of 20-229 kDa were also run. The proteins were resolved by SDS-PAGE on 10% polyacrylamide gels, at 100V. For western blotting the proteins were transferred from the gel to nitrocellulose membrane. The membrane was moistened in water and equilibrated in transfer buffer (25mM Tris base, 250mM glycine, 20% methanol in DW) for 10 min and then placed over the gel. The gel transfer took place overnight at 4°C at 30 V. The proteins transferred on membranes were visualized with ponceau stain for 2-3 min and destained with DW. The membranes were then put in the blocking solution comprising 5% non fat dry milk (NFDM) in 0.01 M PBS (pH 7.4) for 2 hr at room temperature followed by incubation with the primary antibody to vimentin or vinculin (Santa Cruz Biotechnology, USA) at a dilution of 1:100 in 0.01 M PBS containing 5% NFDM at 4°C over night. Normal goat serum was substituted for primary antibody in the negative controls. The membranes were washed for 1 hr, (each wash of 10 min) with the wash buffer (0.01M PBS containing 0.1% Tween 20). The membranes were then incubated with the HRP labeled, rabbit anti-goat secondary antibody (DAKO) for 1 hr at RT at a dilution of 1:1,000 (in the blocking buffer), followed by 6 washes of 10 min each with 0.01M PBS containing 0.1% Tween 20. The proteins were visualized by chemiluminescence. The protein bands on the photographic films were quantified using Gel Pro 3.1 image analysis software.

**Detection of vimentin and vinculin transcripts by biplex RTPCR in testis**—Rat testicular tissue (50-100 mg) slices were homogenized in 1 ml of Trizol in 15ml RNAse free polypropylene tubes with an electric homogenizer and centrifuged at 15,000 rpm for 15 min. Supernatants were transferred to fresh tubes. To this supernatant, 0.2 ml chloroform was added per ml of Trizol reagent, thoroughly mixed and kept at RT for 5 min. The mixture was centrifuged at
12,000 x g for 15 min at 4°C in a tabletop ultracentrifuge. The upper aqueous phase containing the RNA was transferred carefully to a new tube to which 0.5 ml of isopropanol (per 1 ml of Trizol used for initial homogenization) was added. The RNA was allowed to precipitate at RT for 10 min, pelleted by recentrifugation at 12,000 x g for 10 min at 4°C. RNA pellet was washed with 1 ml of 70% ice-cold ethanol, recentrifuged at 7,500 x g for 5 min at 4°C and air-dried. The RNA pellet was dissolved in RNase free diethylpyrocarbonate (DEPC, Sigma, USA) treated water by warming at 65°C for 10 min and stored at -70°C. Integrity of the RNA was checked by resolving it on 1.5% agarose gel. The concentration and purity of the extracted RNA were determined by measuring the absorbance of the samples diluted in DEPC water by warming at 65°C (1:100) at 260 and 280 nm. This RNA was used for RT-PCR. RNA concentration was calculated using the formula, OD at 260 x 0.04 x 100 = μg RNA/μl, where 100 is the dilution factor, 0.04 is the RNA concentration in μg per μl at an OD of 1.

Biplex RTPCR for vimentin—1 μg of the RNA was reverse transcribed at 48°C for 45 min using AMV reverse transcriptase and amplified using Titan one tube RT-PCR system (Roche Diagnostics, Germany). The forward 5'-CCAGATCTATCTTGCGCTCC-3' and the reverse primers 5'-TGAG ACTTCTCAGCACCACG-3' (Bangalore Genie, India) were designed from rat database (gene accession number AA 924259) and gave amplified product of 155 bp. The sense and antisense primers for mouse β-actin used as internal loading control were 5'-CTGGCACACACCTCTTA-3' and 5'-GGGCACAGTGTGGGTGAC-3' (gene accession number NM_007393), respectively which gave the product size of 240 bp. Twenty five cycles were selected for amplification in order to be in the exponential phase. For PCR, reverse transcriptase was inactivated at 94°C for 2 min followed by cDNA denaturation at 94°C for 1 min, primer annealing at 58.5°C for 1 min and primer extension at 72°C for 1.5 min. Final primer extension was done at 72°C for 15 min.

Quantitation—The PCR products in 20 μl of samples were resolved on 2% agarose gel containing ethidium bromide to check the product size. DNA markers were run along with the products. The resolved PCR products were visualized with a UV transilluminator and quantified using Gel Pro 3.1 image analysis software. Data were expressed as the ratio of integrated optical density (IOD) of vimentin/vinculin and β-Actin cDNA products.

Results

Effects of estradiol treatment on serum hormones and sperm counts—Estradiol treatment produced a significant reduction in the serum levels of LH from 1.5±0.71 to 0.17±0.17 ng/ml, FSH from 12.9±4.7 to 5.3±2.05 ng/ml and testosterone from 1.15±0.81 to 0.27±0.16 ng/ml, while estradiol increased from 73.9±27 to 139.37±15.28 pg/ml as compared to control animals. The sperm counts were significantly lowered in the treated rats from 49±13.5 to 35±12 million/cauda.

Effects of estradiol treatment on testicular histology and BrdU labelling (Fig. 1 A-D) — A preponderance of seminiferous tubules with round spermatids could be observed in rat testis after estradiol treatment (Fig. 1A), whereas elongating spermatids were observed in the tubules of control testis (Fig. 1A, inset). A significant reduction was observed in the mean Sertoli cell height from 9.85±0.97 to 6.7±0.97 μm in testicular sections after estradiol treatment (Fig. 1B). In estradiol treated testis, there was disorganization of germ cells in the
seminiferous tubules. Round spermatids were often noticed near the basement membrane instead of towards the lumen of the tubules (Fig. 1C; cf. Fig. 1C: inset: control). BrdU incorporation was observed in germ cells near the basement membrane of the seminiferous tubules of the control testis (Fig 1D, inset). BrdU labelled germ cells were scattered from the basement to the lumen in the tubules of the estradiol treated testis (Fig 1D). BrdU labeling of germ cells in both control and treated testis suggested that germ cell proliferation was maintained during estradiol treatment.

Effects of estradiol treatment on the expression of testicular vimentin and vinculin genes (Fig. 1 E,F)— Estradiol treatment did not produce any significant change in the expression of vimentin or vinculin genes in the testis as seen from ratios of IODs of vimentin to actin bands (1.14±0.09 to 1.26±0.14, n=6) or vinculin to actin bands (0.35±0.12 to 0.48±0.14, n=6) as compared to controls (Fig. 1E, F upper left panel). The suppression of FSH and testosterone after estradiol treatment did not affect the expression of the two cytoskeletal proteins but may have led to

Fig. 1A-D—Effect of estradiol treatment (0.1mg/kg/day × 10days) in rats on the (A) histology of seminiferous tubules showing predominantly round spermatids in treated testis and elongated spermatids in control (inset) (B) Sertoli cell height and serum FSH levels (C) lipid droplets in Sertoli cell cytoplasm (D) incorporation of BrdU in germ cells of control (inset) and treated testis (estradiol treatment at 0.1mg/kg/day × 22days).

Fig. 1 E—Effect of estradiol treatment (0.1mg/kg/day × 10days) on vimentin expression (upper left panel), levels (upper right panel) and localization (lower panel) in rat testis. (a) negative control, (b) Control, (c) treated.
degradation of vimentin, failure of polymerization and collapse of vimentin filaments leading to the disorganization of tubular cytoarchitecture.

Effects of estradiol treatment on the testicular vimentin and vinculin protein levels (Fig. 1 E-F upper right panel)—Specific bands of vimentin 58 kDa and vinculin 130 kDa were identified by western blotting. Estradiol treatment did not produce a significant change in the testicular levels of vimentin (IODs, 11.83±0.27 to 13.33±1.66, n=6) or vinculin (IODs, 2.63±1.96 to 3.29±1.79, n=6) as compared to controls.

Immunolocalization of vimentin and vinculin in testis (Fig. 1Ea,b,c,Fa,b,c) —Vimentin staining was intense and uniform throughout the control rat tubules, radiating as expected, from the base to the lumen (Fig. 1Eb). Vinculin staining was intense and localized as expected to the luminal periphery around the germ cells in control rat testis (Fig. 1Fb). After estradiol treatment immunolocalization of both vimentin and vinculin in testis was observed to be diffuse, suggestive of a collapse of normal organization within the Sertoli cells (Fig. 1Ec; Fig.1Fc).

Discussion
Xenoestrogens have become a matter of serious concern, in view of the hazards these pose to male fertility. Estrogen receptors, known to be expressed throughout the male reproductive tract, are being viewed as mediators of the adverse estrogenic effects of environmental estrogens on spermatogenesis in the testis. However, apart from the well-documented neuroendocrine effects of estrogens, new insights have been gained about their effects on spermatogenesis at the molecular level are relatively few. In the present study, short-term estradiol treatment decreased the peripheral levels of LH, FSH and testosterone, while that of estradiol increased in adult male rats. The neuroendocrine changes occurred concomitant with a significant reduction in sperm counts. Arrest of spermatogenesis did not occur, but short term treatment did lead to disorganization of the cytoarchitecture within the seminiferous tubules. The Sertoli cell height was significantly reduced in estradiol treated tubules. Furthermore, a preponderance of tubules with round spermatids was noticed in estradiol treated testis, whereas elongating spermatids could be observed in control testis.

In rat, spermatogenesis occurs in 14 well defined stages of the germinal epithelium, regulated by testosterone and FSH. The androgen and FSH receptors are expressed in a stage specific manner in the seminiferous epithelium, but their effects at the molecular level remain to be elucidated. Both hormones have been implicated in maintaining the anchoring junctions between the Sertoli and germ cells. Sertoli cells occupy a volume of 17-19% in adult rat testis. The enormous surface area allows these cells to support the development and movement of a vast number of germ cells in a ratio of 1:50 in adult rats. These cells have a well-developed cytoskeleton, which has been implicated in maintaining the changes in shape, transport of organelle, formation of several types of junctions, movement of germ cell lineages and spermiation during spermatogenesis. The expression or organization of the cytoskeletal proteins vimentin, vinculin and actin undergo spatiotemporal changes.
Cyclic changes in the surface area of organelles like mitochondria, rough endoplasmic reticulum, and topographical changes in Golgi apparatus, lysosomes and lipid droplets occur in a stage specific manner in each cycle of the seminiferous epithelium. Stage specific changes in Sertoli cell organelles, also characteristic of FSH and androgen receptors, suggest that these are regulated by FSH and androgen. Suppression of either of the two hormones or disruption of the endocrine axis, affects cytoskeletal proteins. Estradiol-induced suppression of FSH and testosterone has been reported to cause disorganization of the cytoarchitecture within the seminiferous tubules, sloughing and selective apoptosis of differentiating germ cell lineages. Estradiol has also been directly implicated in germ cell proliferation, survival and acrosome biogenesis in spermatids.

In the present study, estradiol-induced reduction in Sertoli cell height, and a diffuse pattern of localization of intermediate vimentin filaments in the Sertoli cells, were suggestive of a reduced capacity of the cells in supporting differentiating germ cells. Sertoli cells undergo a morphological change from type A to B during the stages of spermiogenesis in rats. The intermediate vimentin filaments, which extend from the Sertoli cell nucleus to its apex, have also been reported to vary in length dynamically with the stages of the cycle. The shortest length occurs at the time of spermiogenesis in the androgen dependent stages, implicating this protein in spermiogenesis. Collapse of vimentin filaments has been observed in cryopreserved testis of immature rats concomitant with induction of massive apoptosis. Exposure to toxicants and colchicine has also been shown to lead to collapse of vimentin cytoskeleton concomitant with sloughing and induction of a wave of apoptosis in germ cells. Recent studies in rats wherein suppression of intratesticular testosterone levels with combined testosterone and estradiol treatment led to the collapse of Sertoli cell vimentin filaments have suggested that testosterone could be involved in mediating the observed spermiogenesis related morphological changes in the Sertoli cells. It was suggested that changes in Sertoli cell vimentin filaments induced by androgen deficiency could be a signal for germ cell apoptosis.

In the present study, no change was observed in the expression or levels of vimentin in the whole testis, in spite of reduction in testosterone levels. The results suggest that testosterone may not be involved in vimentin gene expression. On the contrary, estradiol itself could have directly maintained the expression of vimentin. However, the altered profile of vimentin filaments in the Sertoli cells, with staining being localized towards the base of the tubule, was consistent with reported effects of androgen deficiency on the degradation of polymers. In addition to androgen, FSH is also considered to regulate the columnar shape of seminiferous epithelium through phosphorylation of vimentin filaments. The Sertoli cell vimentin filaments are extensively branched in the FSH dependent stages of spermatogenesis when spermatids are elongating. This could signify an increase in the volume of these cells to accommodate the elongating spermatids in crypts. Thus, FSH suppression may also have affected the organization of vimentin filaments in the seminiferous epithelium and thereby Sertoli germ cell adhesions. The breakdown of vimentin filaments due to suppression of testosterone and FSH would have signaled a wave of germ cell apoptosis in the estradiol-treated testis.

Vinculin, the actin binding protein of Sertoli germ cell adhesion junctions, shows staining from nucleus to apex of Sertoli cells at the time of spermiogenesis, in stage VIII of spermatogenesis in rats. It reportedly localizes to step 19 spermatids as well as to residual bodies after spermiogenesis. Lipid droplets increase characteristically after spermiogenesis as a result of digestion of the residual bodies. In the present study, estradiol-induced diffuse pattern of vinculin staining in Sertoli cells (identical to that seen after hypophysectomy), concomitant with massive increase in lipid droplets is suggestive of failure of spermiogenesis, abnormal retention and phagocytosis of elongated spermatids in the Sertoli cells. As in the case of vimentin, no effect was seen on the expression or levels of vinculin in estradiol treated testis, in spite of reduced levels of FSH and testosterone. The results suggest that estradiol could be directly involved in vinculin gene expression. The diffuse vinculin immunolocalization in estradiol-treated testis suggested that suppression of testosterone and FSH affected the peripheral distribution of vinculin in Sertoli cell junctions. The preponderance of stage VII tubules observed in the testis of estradiol-treated rats, characteristic of mid-spermiogenetic arrest, is typical of testosterone withdrawal. Stage synchronization has only been reported after induction of vitamin A deficiency in rats. This observation is interpreted to suggest that estradiol treatment also led to failure of terminal differentiation of spermatids due to changes in Sertoli cell volume.
In the present study, estradiol treatment reduced sperm counts in rats by suppressing serum testosterone and FSH. Withdrawal of FSH is associated with apoptosis of spermatagonia and spermatocytes in rat, while that of testosterone entails loss of spermatocytes, and round and elongated spermatids. Furthermore, withdrawal of gonadotropins reportedly leads to apoptotic loss of germ cells in stages VII-VIII whereas estradiol induced germ cell apoptosis occurs in stages X-II. In view of the suppression of both FSH and testosterone by estradiol, apoptosis would have been induced in spermatogonia, spermatocytes, round and elongating spermatids. Paradoxically, estradiol treatment has also been reported to produce a protective effect in spermatogonia A, preleptotene and metaphase spermatocytes besides being mitogenic to germ cells. In an interesting development, D'Souza et al have recently also reported a significant increase in intratesticular levels of estradiol in rats with a protective effect on germ cell apoptosis, after short-term estradiol treatment. In the present study, incorporation of BrdU in germ cell DNA of both treated and untreated tubules also supported the mitogenic role of estradiol. Thus, arrest of proliferation does not appear to have caused reduction in sperm counts. Rather, scattered BrdU staining in the estradiol treated tubules suggested that collapse of Sertoli cell cytoskeleton and consequent disorganization of tubular cytoarchitecture affected Sertoli cell volume, (reminiscent of changes seen in cryptorchid primate testis), would have induced apoptosis in earlier lineages of differentiating germ cells. Thus, failure to support the differentiation of adequate numbers of earlier germ cells would have been responsible in part, for the estradiol induced reduction in sperm counts.

In conclusion, the decrease in sperm counts produced by short term estradiol treatment is a gradual process which appears to have occurred due to a collapse, but not expression, of Sertoli cell cytoskeleton, altered morphology with reduction in volume, failure to support differentiation of adequate numbers of germ cell lineages, sloughing and failure of spermiogenesis rather than reduction in mitotic proliferation. The study may have important toxicological implications for male infertility.

Acknowledgment

Financial assistance for this project was provided to Ms Pritha Das-Gupta (summer trainee; University of Mumbai, Dept of Biotechnology) for her M.Sc dissertation, by Indian Council of Medical Research, India. The authors acknowledge the gift of reagents for radioimmunoassay of protein hormones from NIADDK, USA. The authors are thankful to Dr. Ikram Khattakat (NIRRH, Mumbai) for the gift of testosterone antiserum. The authors appreciate the technical assistance of Mr H G Pawar, and thank Mr S Mandavkar for handling the animals.

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