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Exposure of adult rats to estradiol valerate induces ovarian cyst with early senescence of follicles

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Abstract

Environmental and therapeutic estrogens are known to play an important role in modulating the reproductive life and pubertal maturation in males as well as in females. Animal studies have shown that exogenously administered estrogen induces follicular cysts. However, the probable mechanisms underlying this abnormal ovarian development and its impact on steroidogenesis have been ill defined. The present study was therefore carried out to understand the ontogeny of ovarian pathology owing to adult estrogenisation. Regularly cycling female Holtzman rats were sacrificed at one week, two weeks, three weeks and four weeks after a subcutaneous administration of 2 mg of estradiol valerate (E_2V). The effect of this supra-physiological estrogen on serum endocrine profiles, development of follicular cysts, follicular apoptosis and expression of markers of folliculogenesis viz., estrogen receptor (ER)-β, inhibin A and progesterone receptor (PR) were studied. Results indicate a temporal augmentation of steroidogenesis, which was associated with induction of follicular cyst with theca cell hyperplasia and induction of apoptosis in the primary and secondary follicles of the ovaries. Immuno-histochemical localization showed an increase in inhibin A with a reduction in ER-β and PR indicating early maturation, poor follicle growth and granulosa cell differentiation. Results indicate that exposure to exogenous estrogen in adulthood can have deleterious effects on the ovarian physiology and endocrinology which may ultimately lead to cystogenesis, loss of follicle pool and early senescence.

Keywords: Estradiol valerate; Cystic follicles; Hyperandrogenemia; Apoptosis

1. Introduction

Estrogens are important for the development of sexual phenotype, reproductive tract morphology and sexual differentiation of the central nervous system. In the course of normal mammalian reproduction, estrogens are believed to play crucial roles in the development of pituitary gonadal axis, ovarian folliculogenesis, ovulation and implantation. Physiologically, estrogen mediates its action through two well-characterized estrogen receptors (ER-α and ER-β), which are transcription factors (Mathews and Gustaffson, 2003). Knockouts of either of these receptors result in compromised reproductive functions such as altered ovarian folliculogenesis, failure of ovulation and corpus luteum formation (Couse et al., 2003). These observations suggest that ERs play a facilitatory role in ovarian follicular development and maturation (Palter et al., 2001; Couse et al., 2005) emphasizing the importance of estrogen and its receptors in the ovarian physiology.

Although, estrogen is recognized as a prime hormone in regulation of female reproductive physiology, exposure of estrogenic compounds clinically or environmentally, impose varied undesired effects on female reproductive system ranging from altered pubertal maturation to infertility (Danzo, 1998; Richter et al., 1998; Holmes and Harrison, 2001). It is now becoming increasingly evident that *in utero* exposure to estrogen leads to developmental abnormalities in reproductive system (Singh, 2005). Extensive animal experimentations have demonstrated that exposure of low doses of androgens via aromatisation to estrogen or low doses of estrogen itself neonatally or prenatally result in long-term reproductive consequences including hyposteroidogenesis, lack of ovulation and suppression of gonadotropins and development of cystic follicles at adulthood (Pinilla et al., 1993; Deshpande et al., 2000; Jefferson et al., 2002). These effects of neonatal estrogenisation are irreversible and show a long lasting effect on ovarian physiology.

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However, besides neonatal exposure, adult females are also exposed to supra-physiological levels of estrogen either endogenously (hyperestrogenemia, estrogen producing tumors) or through environmental contaminants, occupational exposure and also through therapeutics (Matsumoto, 1992; McKenna et al., 1990; Danzo, 1998; Richter et al., 1998). While, the imprinting effects of neonatal exposure to exogenous estrogen have been extensively described, limited data exists on the consequences of estrogenization during adulthood on ovarian physiology and endocrinology. We hypothesize that the long-range effects of estrogenization (if any) in the adults would considerably differ from those observed during developmental exposure, because the regulatory mechanisms and the dynamics of the endocrine axis differs significantly during development as compared to adulthood. Some support to this hypothesis is gained from the observation that neonatal estrogenization leads to anovualtion in adulthood with low levels of estradiol and gonadotropins (Pinilla et al., 1993; Deshpande et al., 2000), while animals exposed to estrogen during adult life show early luteinization of follicles, large corpora lutea and development of cystic follicles along with depletion in follicle numbers over a period of time (Brawer et al., 1986). We have earlier demonstrated that exposure to estrogen in adult life results in ovarian follicular cystogenesis, reduced number of corpora lutea, degeneration of the hypothalamus-arcuate nucleus and impaired hypothalamus–pituitary axis (Brawer et al., 1978; Maitra and Nandedkar, 1994). However, limited information exists on the ontogeny of ovarian pathology and the characteristics of the cystic follicles that occur as a result of adult estrogenization. The effects on steroidogenesis and the molecular basis of disturbed folliculogenesis and cystogenesis are not clearly defined in this system.

To investigate the physiological and molecular basis of ovarian dysfunction in response to supra-physiologic estrogen in adults, the present study was undertaken to explore in detail, the effects of adult exposure to estrogen on ovarian folliculogenesis. In this communication, we present our data on the endocrine profiles, steroidogenesis, ovarian morphology and expression of selected markers involved in folliculogenesis.

2. Materials and methods

The study was carried out after obtaining approval from the institutional animal ethics committee.

2.1. Experimental design

Six- to eight-week-old, cyclic, randomly bred *Holtzman* female rats (wt. 150–200 gm) were used in the study. The animals had free access to pelleted rat food and water and were maintained at a temperature of 22–23 °C, humidity 50–55% and light and dark cycles of 14 h and 10 h, respectively at the experimental animal facility of the institute. Fifty animals displaying at least two consecutive normal 4-day estrus cycles were used in this study. Estrus cycles prior to and after treatment were monitored by daily examination of vaginal smears.

Estradiol valerate (E_2V), an analogue of 17β estradiol was obtained from Sigma (St. Louis, USA). The animals were divided into four experimental groups and one control group ($n = 10$ animals per group). A single dose of 2 mg/animal in olive oil was administered subcutaneously to the experimental group. This dose was chosen as we and others have previously demonstrated that this bolus dose to adult females affects ovarian physiology and results in cystic follicle formation (Brawer et al., 1986; Maitra and Nandedkar, 1994; Lee et al., 2003; Manni et al., 2005). All the animals were injected on the day of estrus and then sacrificed at different time intervals viz., one week, two weeks, three weeks and four weeks post exposure. The control animals were treated identically except that they were injected with equivalent amounts of vehicle only. All groups of animals were always sacrificed between 09.30 a.m. and 10.00 a.m. Daily vaginal smears were taken to check the estrus cyclicity that was assessed by analysis of the relative proportion of leukocytes, epithelial and cornified cells (Rosa-E-Silva et al., 2003). The animals were euthanized under ether anesthesia and blood was collected by cardiac puncture. Serum was separated and frozen at −20 °C for hormonal estimations. Both the ovaries were carefully dissected and freed from the surrounding fat. Randomly selected ovaries from each experimental group ($n = 10$ ovaries from five animals) and a control group ($n = 10$ ovaries from five animals) were fixed in 10% buffered formalin and processed for routine paraffin embedding and sectioning. Rest of the ovaries ($n = 10$ ovaries from five animals) was snap frozen in liquid nitrogen and stored at −70 °C for RNA extraction.

2.2. Histology

Ovarian tissue fixed in 10% neutral buffered formalin was dehydrated through grades of alcohol, cleared in xylene and embedded in paraffin wax. Five micrometres thick sections from paraffin embedded ovarian tissue were cut and stained with Mayer's hematoxylin and eosin. Random sections from each ovary were used for histological studies. Two independent observers evaluated all the slides microscopically in a blinded manner. The follicles were classified according to the standard morphological criteria (Kishi and Greenwald, 1999). The cystic follicles were defined as follicles that were as large as antral follicles with attenuated granulosa cell layer and large antral cavity with thickened theca layer (Lara et al., 2002). The size of corpora lutea (CL) and the thickness of theca cell (TC) layers of different follicles were compared between control ($n = 3$) and E_2V exposed (two-week post exposure) animals ($n = 3$) using the morphometric analysis software from Biovis. In all, 31 CL and TC layers from 40 different follicles were estimated for size in the experimental group (two-week post exposure group), whereas 36 CL and TC layers from 59 different follicles were studied in control group.

2.3. Measurement of circulating levels of serum gonadotropins and steroids

Serum LH and FSH were assayed using standard Radio Immuno Assay kits provided through the NIADDK National Pituitary Agency. The standard curve for LH (NIADDK-Rat-LH-RP-2) and FSH (NIADDK-Rat-FSH-RP-2) ranged from 10 ng/ml to 12.5 pg/ml. All the samples were analysed in duplicates. The inter assay and intra assay coefficients of variation were 9% and 6% for LH and 10% and 6% for FSH. Serum levels of androstenedione (A4) were measured by radioimmunoassay (RIA) as described previously (Tanavde and Maitra, 2003). Antibody for the same was kindly provided by Dr. S. G. Hillier (UK). The standard curve for A4 ranged from 5 ng/ml to 39 pg/ml and the inter assay and intra assay coefficients of variation were 11% and 5.5%. Serum progesterone (P4) and estradiol (E2) were assayed by RIA as described previously (Tanavde and Maitra, 2003) using antisera from ICN Biomedical, Germany. The assay sensitivity, the inter assay and intra assay coefficients of variation for P4 was 6.25 pg/ml, 12% and 10%, respectively. The assay sensitivity, the inter assay and intra assay coefficients of variation for E2 was 3.9 pg/ml, 10% and 6%, respectively. Cross reactivity assay was carried out with E2 antisera and E_2V as a standard so as to determine the possibility of estimation of E_2V from serum.

2.4. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen ovaries using Trizol reagent (Invitrogen, Germany) according to manufacturer's protocol. All RNA samples were treated with 0.2 μgm/ml of RNAse free DNAse I (Sigma) to remove contaminating DNA. RT-PCR was carried out according to the protocol detailed previously (Modi et al., 2005). Briefly, 2 μg of total RNA was reverse transcribed using oligo dT primers and M-MuLV as enzyme (Promega Corp. Medison, WI) in a total volume of 20 μl. Two microlitres of the single stranded cDNA