Clomiphene citrate potentiates the adverse effects of estrogen on rat testis and down-regulates the expression of steroidogenic enzyme genes

Shilpa Bharti, M.Sc.,[a,b] M. M. Misro, Ph.D.,[a] and Umesh Rai, Ph.D.[b]

[a] Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka; and [b] Department of Zoology, University of Delhi, New Delhi, India

Objective: To investigate the antiestrogenic effect of clomiphene citrate (CC) in male rats estrogenized with estradiol-3-benzoate (EB).
Design: Prospective experimental study.
Setting: Laboratory.
Animals: Adult male albino rats (Holtzman strain).
Intervention(s): CC was given alone or in combination with EB.
Main Outcome Measure(s): Testicular function and steroidogenic enzyme gene expression were evaluated in control versus treated groups.
Result(s): EB after 30 days of treatment induced a rise in TUNEL-positive germ cells adversely affecting spermatogenesis with complete absence of elongated spermatids or sperms. CC alone had only a moderate effect. In contrast, CC+EB synergistically inflicted more adverse effects as apoptotic germ cells per tubule rose further. Significant down-regulation in expression of testicular steroidogenic enzyme genes StAR, p450scc, 3β-HSD, and p450c17 was observed. In the EB-alone group, aromatase gene expression in the testis was up-regulated but reversed in brain and liver tissues. CC alone had little modulatory effect on aromatase expression. On the other hand, CC+EB countered the EB-induced rise of aromatase expression in the testis.
Conclusion(s): The above findings indicate that CC in the presence of estrogen synergistically potentiates more adverse effects in testis, inhibiting expression of upstream steroidogenic enzyme genes and leading to disruption of steroidogenesis. (Fertil Steril® 2013;99:140–8. ©2013 by American Society for Reproductive Medicine.)
Key Words: Clomiphene citrate, estradiol-3-benzoate, testis, antiestrogen, steroidogenic enzyme, gene expression

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/bhartis-clomiphene-citrate-estrogen-testis/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

The role of estrogen in adult testicular function is very critical, because complete absence of its synthesis, as seen in ArKO (aromatase knockout) male, is associated with abnormal development of round spermatids with lesions (1, 2). On the other hand, excess of estrogen administered in mice during fetal or neonatal period, has been implicated in several male reproductive disorders, such as cryptorchidism, epididymal defects, impaired fertility, and testicular cancer (3). It is thus recognized that optimal estrogen availability is essential for maintaining normal testicular function in adults. Germ cell development relies on highly coordinated action of various reproductive hormones, including gonadotropins, which are the prime regulators of both spermatogenesis and steroidogenesis (4). Excess estrogen in

Received May 29, 2012; revised and accepted August 22, 2012; published online September 19, 2012.
S.B. has nothing to disclose. M.M.M. has nothing to disclose. U.R. has nothing to disclose.
Supported by Council for Scientific and Industrial Research and National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka, New Delhi, India.
Presented at the annual congress of the Society of Andrology: India, Indian Institute of Technology, New Delhi, December 10–12, 2011.
Reprint requests: M. M. Misro, Ph.D., Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka, New Delhi-110067, India (E-mail: mm_misro@yahoo.com).

Fertility and Sterility® Vol. 99, No. 1, January 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2012.08.050

adults, however, down-regulates gonadotropins and alters the entire hormonal milieu leading to impairment of spermatogenesis and apoptotic induction in developing germ cells (5, 6). Aromatase is the key converting enzyme for estrogen synthesis, and the modulation of its expression would have a direct bearing on the estrogen availability within the testis.

Besides the hormone per se, even the lack of functional estrogen receptor alpha (ERα) leads to infertility in ERαKO male mice (7–9). Similarly, when estrogen receptor in the rat was blocked by ICI 182,780 for 100–150 days, it resulted in testicular atrophy and infertility (10). In contrast, clomiphene citrate (CC) has been described to possess both estrogen agonist as well as antagonistic properties. It is reported that in presence of 17β-E_2 and mediated via ERα, CC behaved as an agonist when estrogen was in low concentrations and as an antagonist when estrogen was in high concentrations. But via ERβ, it primarily acted as an estrogen antagonist regardless of the concentration of estrogen in the surrounding (11).

CC is an analogue of nonsteroidal estrogen, a triphenylethylene derivative distantly related to diethylstilbestrol, and has been proved very effective in the induction of ovulation in infertile women (12). It acts as a selective estrogen receptor (ER) modulator, similarly to tamoxifen and raloxifene. All three drugs are competitive inhibitors of estrogen binding to estrogen receptors and have mixed agonist and antagonist activity depending on the target tissues (13). Estrogen receptors are activated by a range of ligands, including these selective ER modulators. And, like other nuclear receptors, ligand-bound ERs act as dimers to regulate transcriptional activation (14) to support estrogen agonistic or antagonistic action. The estrogen antagonistic property of CC in vivo has been debated in the past, and not much information is available on its mode of action in human males when given alone or in combination with exogenous estrogens. CC is also very much in use for the treatment of male infertility, because it exerts an effect stimulating spermatogenesis (15, 16). However, there are contradictory reports that question the beneficial effects of CC in improving male fertility (17, 18). Given the potential importance of estrogen in male reproductive function in adults and that of CC as a debatable spermatogenesis-stimulating agent, the present study was designed to investigate the effects of this estrogen modulator (CC) on testicular function, germ cell apoptosis, and expressions of steroidogenic enzyme genes and aromatase under excess-estrogen conditions.

MATERIALS AND METHODS

Animals, Treatment, and Sample Preparation

Adult male albino rats (Holtzman strain), weighing 200–250 g, were maintained under controlled temperature (25 ± 2°C) and constant photoperiodic (12 h light–12 h dark) conditions with food and water ad libitum. Animal experiments were carried out under strict compliance with the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India, and institutional guidelines for animal care. Requisite approval from the Institutional Animal Ethical Committee was obtained for the study. The animals (n = 24) were divided into four groups. E_2-3-benzoate (EB; Sigma Chemical Co.) and CC (Sigma Chemical Co.) were dissolved in isopropanol and later suspended in olive oil to make a homogeneous suspension. The suspensions were kept overnight at 37°C to allow the alcoholic portion to evaporate, following which they were administered intramuscularly. The details of drug administration as carried out are described below.

Group 1: Olive oil (100 μL, vehicle control) every 5th day

Group 2: EB (75 μg in 100 μL) every 5th day

Group 3: CC (5 mg in 100 μL) every 5th day

Group 4: CC (5 mg) + EB (75 μg) in 100 μL every 5th day

Starting from day 0 when the drug was administered, subsequent interventions were carried out every 5th day until 15 or 30 days. Thus, on the whole, there were two sets of animals in different groups (n = 6 per group) as detailed above which were maintained separately, coinciding with the duration of drug treatments. The dose of EB was selected on the basis of reported findings (19) and subsequent studies (unpublished observations) carried out in our laboratory. A single dose of EB was considered to be sufficient because the aim of the present study are to maintain estrogen excess conditions in the testis and subsequently investigate its modulation on the expressions of steroidogenic enzyme genes. CC dose and duration of intervention was finalized based on observations in animal studies reported earlier (20). The half-life of CC has been reported to be 5–7 days (11). Therefore, it was administered every 5th day along with EB. At the end of 15 and 30 days' treatment, the animals were anesthetized under ether. After blood collection from the tail vein, the animals were killed with an overdose of the same anesthesia and the testes dissected, weighed, and divided into two equal groups. One group was immediately fixed in buffered formalin for histologic analysis and the other frozen in liquid nitrogen and stored at −80°C until used for real-time polymerase chain reaction (PCR), Western blotting, and estimations of intratesticular T or E_2. Besides testes, brain and liver were also collected for analysis of aromatase gene expression. Serum was separated by centrifugation at 5,000*g* for 15 minutes and stored at −20°C until assayed for LH, T, and E_2.

Testes Histology and Quantification of Spermatogenesis

After 4 hours in buffered formalin, the two poles of the testis were cut with a fine blade and left for 24 hours at 4°C. Tissue was washed off the fixative, dehydrated in upgraded series of ethanol, cleared in xylene, and finally embedded in paraffin at 60°C. Sections (4 μm) were cut with the use of a semiautomatic microtome (Leica Microsystems) from paraffin-embedded blocks precooled in ice and layered on poly-L-lysine coated glass slides. The tissue sections were deparaffinized, cleared with xylene, and rehydrated before being stained with hematoxylin and eosin and mounted in DPX (distrene, plasticiser, xylene). Testis sections were examined under microscope (Nikon Eclipse E600) and photographed with the help of an image analyzer. Quantification of the spermatogenesis in the testes of control and treated rats (n = 6) was carried out as previously