Chapter 4

To Find out the Effects of Estrogen Induced Modulation of Expression of Aromatase and other Steroidogenic Enzyme during Hypospermatogenesis using a Mammalian Model
4.1. Introduction

The role of estrogen in adult testicular function is very critical as complete absence of its synthesis as seen in ArKO (Aromatase knock out) male was associated with abnormal development of round spermatids with lesions (O’Donnell et al., 2001; Robertson et al., 2002). On the other hand, excess of estrogen administered during fetal or neonatal period, has been implicated to several male reproductive disorders, such as, cryptorchidism, epididymal defects, impaired fertility and testicular cancer (Gill et al., 1979). 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 (Parvinen, 1982). Excess estrogen in adults however, down regulates gonadotropins and alters the entire hormonal milieu leading to impairment of spermatogenesis and apoptotic induction in developing germ cells (Chaki et al., 2006; Kaushik et al., 2010). 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, knock out) male mice (Lubahn et al., 1993; Eddy et al., 1996; Hess et al., 1997). Similarly, when estrogen receptor in the rat was blocked by ICI 182,780 for 100-150 days, it resulted in testicular atrophy and infertility (Oliveira et al., 2001). In contrast, other anti-estrogens, like clomiphene citrate (CC) have been described to possess both agonist as well antagonistic properties. In conditions of complete absence of estrogens, it is likely to induce estrogenic effects but with the presence of estrogens in the environment, its action would be primarily antagonistic (Sorbie and Perez-Marrero, 1984). Accordingly, clomiphene citrate has been used in treating male infertility as it exerts its effect stimulating spermatogenesis by raising endogenous serum LH, FSH and testosterone levels (Patankar et al., 2007). The suggested mechanism of action is blockage of estradiol receptors through the estrogen antagonistic action of CC (Ronnberg et al., 1981). However, there are other contradictory reports in which CC was not considered as a useful drug for treatment of male infertility (Sokol et al., 1998). The expression of enzyme aromatase is very critical in such situations which have never been studied in conjunction in the past. 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 the this estrogen modulator (CC) on testicular
function and aromatase expression under estrogen excess conditions.

4.2. Experimental Plan

Adult male albino rats (Holtzman strain), weighing 200-250g, 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 Committee for the Purpose of Control and
Supervision of Experiments on Animals (CPCSEA), India, and Institutional guidelines for
animal care. The animals (n=24) were divided into four groups. Estradiol 3-benzoate
(Sigma Chemical Co., St Louis, MO, USA) and clomiphene citrate (Sigma Chemical Co.,
St Louis, MO, USA) were dissolved in isopropanol and later suspended in olive oil to make
a homogenous suspension. The suspensions were kept overnight at 37°C to allow the
alcoholic portion to evaporate following which they were administered intramuscularly for
15 and 30 days. The details of the administration as carried out are described below.

Group I : Olive oil (100 µL, vehicle control) / 5th day
Group II : EB (75 µg/rat in 100 µL) / 5th day
Group III : CC (5 mg/rat in 100 µL) / 5th day
Group IV : CC (5 mg/rat in 100 µL) / 5th day + EB (75 µg/rat in 100 µL) / 5th day

The dose of EB was selected on the basis of the previous studies carried out in our
laboratory (Kaushik et al., 2010). CC dose and duration of intervention, on the other
hand, was finalized based on the observations reported earlier (Flickinger, 1997). At
the end of 15 and 30 days treatment, the animals were bled and sacrificed under ether
anesthesia. Testis, were dissected, weighed and divided in two equal groups. One group
was immediately fixed in buffered formalin for histological analysis while the other was
frozen in liquid nitrogen and stored at -80°C till utilized for real time PCR, western
blotting and in the estimations of intratesticular testosterone or estradiol. Besides testis,
brain and liver were also collected for analysis of aromatase gene expression. Serum
was separated by centrifugation at 5000 x g for 15 min and stored at -20°C till assayed
for LH, testosterone and estradiol.
4.3. Material and Methods

The details of the material and protocol used for the experiments have been listed in the preceding chapter, Chapter 2.

4.4. Results

4.4.1. Effects on Spermatogenesis

Exogenous EB administration adversely affected spermatogenesis following 15 (Fig. 1B) and 30 d (Fig. 2B) of treatment but the severity of the effect was more pronounced in the longer exposure group. Compared to controls (Figs. 1A, 2A), CC alone moderately impaired spermatogenesis (Figs. 1C, 2C). On the other hand, spermatogenesis was found severely affected both at 15 (Fig. 1D) and 30 d (Fig. 2D) exposure when EB was co-administered with CC. Sperms were completely absent after 30 d treatment in rats treated with either EB alone or EB+CC regimen. Subsequently, when the impact of estrogen excess on germ cell numbers was compared with that induced by estrogen (EB) plus antiestrogen (CC), an identical pattern of decline in primary spermatocytes and round spermatids was documented; the latter group, however, displaying far less number of cells than the former (Fig. 3A, B).

4.4.2. Apoptotic Induction of Germ Cells

TUNEL positive germ cells were increasingly resolved in the seminiferous epithelium of all treated groups as compared to controls (Fig. 4A, 5A). However, germ cell apoptosis/seminiferous tubule was at its highest in the combination, EB+CC treatment (Fig. 4D, E and Fig. 5D, E) group irrespective of the duration of treatment. Estrogen alone (Fig. 4B, E and Fig. 5D, E) on the other hand, demonstrated a higher apoptotic induction of germ cells (p<0.05) compared to clomiphene citrate alone (Fig. 4C, E and Fig. 5C, E).

4.4.3. Alterations in the Levels of Reproductive Hormones

In an effort to find the answer on the severity of impact on spermatogenesis and apoptotic transformation of germ cells in the EB+CC group, LH, testosterone and estradiol were measured both in the serum and testis extracts. CC alone was able to
significantly bring down the serum LH levels only after 30 d of administration (Fig. 6A). The decline in the LH levels, however, was more acute in the other two treated groups with the EB+CC group again showing a more drastic attenuation compared to the EB alone group. With respect to the decline in testosterone levels in the serum (Fig. 6B) or intra-testicular (Fig. 6C), the trend was very much identical as that of LH.

Estradiol, serum or intratesticular levels, on the other hand, tend to remained unaltered by 15 d (Fig. 7A, B) but increased significantly beyond control levels after 30 d of CC alone treatment. However, EB+CC seemed to enhance both the serum and intra-testicular estradiol levels (Fig. 7A, B). The increase in intra-testicular estradiol was significant (p<0.05) compared to EB alone treatment group at the end of 30 d of treatment.

4.4.4. Expression of StAR and Selected Steroidogenic Enzyme Genes in Testis

Under estrogen excess conditions, StAR and three of the upstream steroidogenic enzyme genes in the testis namely P450scc, 3β-HSD and P450c17 were down-regulated both in their respective protein (Fig. 8 and Table 1, 2) and transcript (Fig. 9) levels. CC supplementation results further decline in the expression levels of these enzymes at the end of 30 d treatment (Fig. 8 and table 1, 2).

Interestingly for the same duration, clomiphene alone also demonstrated identical down-regulation of expression comparable to EB alone or EB+CC combined (Figs. 8, 9 and Table 1, 2) regimen group. But, at the end of 15 d of treatment, it showed no difference in the expression levels when compared with vehicle treated controls. The protein and transcript levels other downstream steroid enzyme genes like 17β-HSD and 5α-reductase, did not demonstrate any change (Figs. 8, 9 and Table 1, 2).

4.4.5. Aromatase Expression in Testis and Peripheral Tissues (Liver and Brain)

Role of exogenous EB and CC was examined in the regulation of aromatase expression in the testis and other peripheral tissues. It was observed that both the compounds whether given alone or in combination were ineffective in altering aromatase expression (protein/transcript) following 15 days of administration in liver and brain (Fig. 10). Similarly, aromatase expression remained completely unchanged even when
CC intervention alone was extended from 15 to 30 d. In comparison, following EB treatment for 30 d, testicular aromatase demonstrated a significant (p<0.001) up-regulation (Fig. 10A, D) of expression (protein as well transcript) which coincided with its down-regulation in liver (p<0.001) and brain (p<0.01) tissues (Fig. 10B,C) at the same time. Surprisingly, testicular aromatase in the EB+CC group revealed no change in expression compared to control levels. In the same group, however, aromatase expression in liver and brain tissues (Fig. 10B, C) revealed significant down-regulation.

4.5. Discussion

The present findings establish that in the adult male the estrogenic potency of clomiphene citrate (CC) combined with estrogen (EB) induces a synergistic effect inhibiting spermatogenesis at a much faster rate and simultaneously pushing more number of germ cells towards apoptosis. Impaired spermatogenesis due to estrogenization in all the treated groups was associated with significant down-regulation in the expression of the three upstream steroidogenic enzymes and StAR protein in the testis. The antiestrogenic potency of CC was only evident in checking the rise of aromatase expression in the testis and restoring the same back to control levels in the EB+CC treated rats.

Clomiphene citrate is an analog of nonsteroidal estrogen, a triphenylethylene derivative distantly related to diethylstilbestrol and has been proved very effective in the induction of ovulation in infertile women (Kamath and George, 2011). It acts as a selective estrogen receptor (ER) modulator, similar to tamoxifen and raloxifene. All three drugs are competitive inhibitors of estrogen binding to estrogen receptors and have mixed agonist and antagonist activity depending upon the target tissues (Cosman and Lindsay, 1999). 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 (Heldring et al., 2007) in a way supporting either estrogen agonist or antagonistic action. However, the estrogen antagonistic property of CC has been debated in the past and not much information is available on its mode of action in adult male when given alone or in combination with exogenous estrogens. In a recent study it was shown that only in male subjects, the relative urinary
concentrations of testosterone, epitestosterone and 4-androstenedione were significantly altered generally after the second day of CC administration. No significant changes, however, were recorded in both sexes on the luteinizing hormone and follicle-stimulating hormone levels (Mazzarino et al., 2011). These data partially confirm our present findings which documented least alterations in serum LH concentrations following initial 15 days of CC administration. But, substantial decrease in serum LH levels (p<0.01) was observed only after a prolonged (30 d) drug exposure (Fig. 6A). The significant decline in serum- or intra-testicular testosterone during the same period was also consistent with the attenuation in LH levels as presently observed (Fig. 6B, D).

Estrogen excess leading to attenuation in the gonadotropin and testosterone levels subsequently affected spermatogenesis (Fig. 2B and Fig.3) as previously demonstrated (Kaushik et al., 2010). However, given in combination with CC, the impact on spermatogenic inhibition was much more acute as the number of prevailing germ cells demonstrated significant reduction even after 15 d of CC+EB exposure (Fig. 1D and Fig. 3A). An identical trend in the induction of germ cell apoptosis was simultaneously observed. The significant rise in germ cell apoptosis in the combination treatment (EB+CC) group (Fig. 4D, E and Fig. 5D, E) may be directly related to a drastic decline in the intra-testicular levels of testosterone (Fig. 6C) as previously reported (Chaki et al., 2006).

Earlier it was reported from our laboratories that complete sperm suppression induced by dienogest and testosterone undecanoate combination regimen was associated with downregulation of expression of upstream steroidogenic enzyme genes, p450scc, 3βHSD and p450c17 and the StAR protein in the testis (Meena et al., 2012). Administration of EB has already been reported to induce down-regulation of expression of testicular steroidogenic enzyme genes responsible for testosterone production in the adult rat (Sakaue et al., 2002). High intra-testicular estrogen was also reported to be associated with such downtrend in the expression levels of these enzyme genes (Balasinor et al., 2010). Though the attenuation in the expression of the above genes was very much similar in the present study (Fig.8 and Fig.9), it cannot be directly correlated to a complete spermatogenic arrest in all the treated groups. CC treatment otherwise resulted a partial impairment of spermatogenesis but led to a significant decline both in protein (Fig.8) and transcript (Fig. 9A, B, C, D) levels of these genes in the testicular tissue.
The concentration of estrogen in the testis and rete testis fluid far exceeds the concentration in the serum of many species which suggest a pivotal role of the hormone in testis and epididymis function (Hess, 2000; Nilsson et al., 2001). Estrogen, at a critical concentration is essential for spermatogenesis and thus a delicate balance of testosterone to estradiol is being maintained inside the testicular milieu (O’Donnell et al., 2001). Exogenous EB administration probably destabilizes this threshold primarily as a result of increase in intratesticular estrogen concentration (Fig.7B). The process gets further accentuated with the down-regulation of LH leading to a decline in Leydig cell steroid synthesis and testosterone availability (Fig. 6A, B, and C). The present findings revealed a significant (p<0.001) rise in serum and intra-testicular estrogen following both CC alone and EB+CC treatment (Fig. 7A, B). Though the increase in the serum levels of estrogen following CC treatment has been reported before (Shoham et al., 1990), the exact mechanism that positively modulates such a synthesis is not very clear. In normal adult rats, the concentration of estrogen in interstitial tissue is reported 9 times higher than that of the seminiferous tubules (de Jong et al., 1974). On the other hand, aromatase activity in germ cells of the adult rodent is equal to or higher than the aromatase activity in Leydig cells (Levallet et al., 1998; Nitta et al., 1993). This supports the contention that germ cells besides the Leydig cells do contribute to the pool of estrogen required for normal testicular function (Kelch et al., 1972). The present findings, however, demonstrate altogether a different picture as CC alone following 30 d of treatment induced a significant decline in the number of prevailing germ cells (Fig. 2C, 3B) but a rise in intra-testicular estrogen levels (Fig. 7B). This discrepancy is hard to explain at this stage and requires further investigation as the expression of aromatase both at the testis and peripheral tissue levels (liver and brain) remained unaltered following CC treatment in comparison with vehicle treated controls (Fig.10).

Aromatase, the microsomal member of the cytochrome p450 superfamily, catalyzes the rate-limiting step in estrogen biosynthesis. Aromatase gene (Cyp19) expression in the testis utilizes a proximal promoter, promoter II (Simpson and Davis, 2001). Despite a varied tissue distribution, its expression is precisely controlled in males (Carreau et al., 2004). It is advocated that there are different mechanisms of regulation of Cyp19 gene expression. This is for the first time in the present study an attempt has been made to understand the way the modulation of aromatase gene expression occurs in the testis.
and peripheral tissues under the influence of either estrogen or ER modulators like CC, given alone or in combination. Excess estrogen was seen negatively influencing aromatase expression in liver and brain while significantly up-regulating the same in the testis. The uptrend in the aromatase expression in the testis of EB treated rats is fully countered with CC co-administration (Fig. 10A and D). Aberrant expression of aromatase has been reported in testicular tumors (Leydigia or seminoma) which resulted rise in estradiol production and disruption of spermatogenesis (Shozu et al., 1998). Since CC treatment negatively modulated estrogen induced Cyp19 expression in the testis and also in the other tissues like liver and brain (Fig. 10B, C, E and F), the significance of its therapeutic use in such cases needs to be further evaluated.

It is believed that as a nonsteroidal antiestrogen, CC competitively binds to estrogen receptors in the pituitary and hypothalamus. This blocks the action of normally low levels of estrogen present in men and induces increased secretion of GnRH leading to a rise in gonadotropin secretion which essentially proved to be helpful in the treatment of male infertility (Zaman et al., 2009). In the male, however, pituitary LH secretion is primarily regulated by circulating estrogen levels rather than testosterone. It is basically estrogen not testosterone which exerts negative feedback on LH release (Finkelstein et al., 1991; Vanderschueren and Bouillon, 2000; Pitteloud et al., 2008; Kumar et al., 2011). CC being an estrogen modulator, in our studies behaved more like an estrogen agonist rather than an antagonist. Accordingly, CC either alone or combined with EB negatively regulated serum LH levels in the treated animals (Fig. 6A) and adversely impacted spermatogenesis (Fig.1 and 2). The present findings thus support the contention that a more carful approach needs to be adopted for use of CC in treatment of male factor infertility.

Despite the abundance of published literature on the response of the testis and spermatogenesis to either estrogen deprivation or estrogen treatment, the exact role of estrogen or estrogen receptor modulators in spermatogenesis and its association to the extent of regulation in the expression of steroidogenic enzyme genes in the testis remain unclear. The above findings establish that being an ER modulator, CC synergistically potentiates the adverse effects of estrogen on the testis. The estrogen antagonistic action, however, was found solely limited to countering the estrogen induced up-regulation of aromatase expression in the testis as presently observed.
Fig. 1: Representative testis sections stained with H & E after 15 d of drug treatment to rats. (A) Vehicle control depicts normal spermatogenesis while impaired spermatogenesis is seen with (B) EB, (C) CC and (D) EB+CC treatment (n=6/group). X400

Fig. 2: Representative testis sections stained with H & E after 30 d of drug treatment to rats. (A) Vehicle control depicts normal spermatogenesis. While (C) CC treatment induces partial arrest of spermatogenesis, it is completely arrested with either (B) or (D) EB+CC treatment (n=6/group). X400
Fig. 3: Quantitation of germ cell numbers per tubule (Mean ± SD) from 20 randomly selected seminiferous tubules in each testis section of control versus treated rats for 15 d (A) and 30 d (B). (B) A significant (***p<0.001) decrease in number of germ cells is observed in rats treated with EB, CC or EB+CC treatment for 30 d. *p<0.01, **p<0.001, ***p<0.001 compared to vehicle treated. "p<0.05, ""p<0.01, "###p<0.001 compared to CC alone. $p<0.05$ compared to EB alone.
Fig. 4: Representative testis section showing TUNEL-positive cells/tubule from 10 randomly selected seminiferous tubules of rats. (A) Vehicle treated control, (B), EB alone, (C), CC alone and (D), EB+CC treatment for 15 d. Note the rise in TUNEL positive cells (→) in testis sections of treated rats (E). *p<0.001 compared to vehicle treated control, #p<0.05 compared to CC alone, $p<0.05 compared to EB alone. X400
Fig. 5: Representative testis section showing TUNEL-positive cells from 10 randomly selected seminiferous tubules of rats. (A), represents vehicle treated control, (B), EB alone, (C), CC alone and (D), EB+CC treatment for 30 d. Note the rise in TUNEL positive cells (→) in testis sections of treated rats (E). *p<0.001 compared to vehicle treated control, #p<0.05 compared to CC alone, $p<0.05 compared to EB alone. X400
Fig. 6: LH and testosterone (serum and intra-testicular, IT) in rats treated with EB, CC or EB+CC for 15 and 30 d. Significant (**p<0.001) decline in (A) LH and (B, C) testosterone was observed in all rats following 30 d treatment. *p<0.01, **p<0.001, ***P<0.001 compared to vehicle treated. #p<0.05, ##p<0.01, ###p<0.001 compared to CC alone. $p<0.05 compared to EB alone.

Fig. 7: Estradiol levels (serum as well intratesticular, IT) from rats treated with EB, CC or EB+CC for 15 and 30 d. Significant (**p<0.001) increase (A) in serum and (B) IT levels was observed in all rats after 30 d treatment. *p<0.01, **p<0.001, ***p<0.001 compared to vehicle treated. #p<0.05, ##p<0.01, ###p<0.001 compared to CC alone. $p<0.05 compared to EB alone.
Fig. 8: Western blot (n=3) analysis depicts the down-regulation of StAR and steroidogenic enzyme genes, p450sc, 3βHSD, p450c17 after 15 and 30 d of EB or EB+CC treatment. CC treatment for 30 d only revealed an identical decline in expression. Protein levels of 17βHSD and 5α reductase remained unchanged.

Fig. 9: Real time PCR analysis depicts the down-regulation of transcripts levels of StAR and steroidogenic enzyme genes, p450sc, 3βHSD, p450c17 after 15 and 30 d of EB or EB+CC treatment. Identical attenuation is seen with CC after 30 d treatment only. Transcript levels for 17βHSD and 5-α reductase remained unchanged in all the treatment groups. *p<0.01, **p<0.001, ***p<0.001 compared to vehicle treated. $p<0.05, ##p<0.01, ###p<0.001 compared to CC alone. $p<0.05 compared to EB alone.
### Table 1: Densitometric analysis of testicular tissue after 15 days of treatment showing ratio of StAR and steroidogenic enzyme protein to β-actin expression ± SD.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Protein name</th>
<th>Control</th>
<th>EB</th>
<th>CC</th>
<th>EB+CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>StAR</td>
<td>0.865 ± 0.039</td>
<td>0.544 ± 0.030</td>
<td>0.682 ± 0.030</td>
<td>0.338 ± 0.031</td>
</tr>
<tr>
<td>2</td>
<td>P450scc</td>
<td>0.976 ± 0.081</td>
<td>0.603 ± 0.092</td>
<td>1.06 ± 0.043</td>
<td>0.581 ± 0.063</td>
</tr>
<tr>
<td>3</td>
<td>3β-HSD (Type I)</td>
<td>0.689 ± 0.060</td>
<td>0.416 ± 0.035</td>
<td>0.712 ± 0.029</td>
<td>0.490 ± 0.055</td>
</tr>
<tr>
<td>4</td>
<td>P450c17</td>
<td>0.945 ± 0.01</td>
<td>0.553 ± 0.052</td>
<td>1.051 ± 0.050</td>
<td>0.652 ± 0.061</td>
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<tr>
<td>5</td>
<td>17β-HSD (Type I)</td>
<td>1.07±0.053</td>
<td>1.065 ± 0.062</td>
<td>1.051 ± 0.050</td>
<td>1.095 ± 0.081</td>
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<tr>
<td>6</td>
<td>5α-reductase (Type II)</td>
<td>0.789±0.060</td>
<td>0.778 ± 0.050</td>
<td>0.726 ± 0.063</td>
<td>0.749 ± 0.061</td>
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<tr>
<td>7</td>
<td>P450 aromatase</td>
<td>0.845±0.068</td>
<td>0.866 ± 0.040</td>
<td>0.812 ± 0.051</td>
<td>0.855 ± 0.042</td>
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*p<0.05, **p<0.01, ***p<0.001 compared to vehicle treated control.

*p<0.05, **p<0.01 compared to CC alone.

*p<0.05 compared to EB alone.

### Table 2: Densitometric analysis of testicular tissue after 30 days of treatment showing ratio of StAR and steroidogenic enzyme protein to β-actin expression ± SD.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Protein name</th>
<th>Control</th>
<th>EB</th>
<th>CC</th>
<th>EB+CC</th>
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<tbody>
<tr>
<td>1</td>
<td>StAR</td>
<td>1.088 ± 0.094</td>
<td>0.561 ± 0.032</td>
<td>0.585± 0.041</td>
<td>0.301 ± 0.026</td>
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<tr>
<td>2</td>
<td>P450scc</td>
<td>0.743 ± 0.029</td>
<td>0.323 ± 0.121</td>
<td>0.471 ± 0.050</td>
<td>0.303 ± 0.028</td>
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<tr>
<td>3</td>
<td>3β-HSD (Type I)</td>
<td>1.625 ± 0.088</td>
<td>0.523 ± 0.028</td>
<td>0.554 ± 0.052</td>
<td>0.401 ± 0.026</td>
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<tr>
<td>4</td>
<td>P450c17</td>
<td>1.065 ± 0.062</td>
<td>0.413 ± 0.049</td>
<td>0.613 ± 0.018</td>
<td>0.473 ± 0.064</td>
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<tr>
<td>5</td>
<td>17β-HSD (Type I)</td>
<td>0.818 ± 0.013</td>
<td>0.814 ± 0.014</td>
<td>0.816 ± 0.021</td>
<td>0.810 ± 0.022</td>
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<td>6</td>
<td>5α-reductase (Type II)</td>
<td>0.554 ± 0.052</td>
<td>0.516 ± 0.035</td>
<td>0.523 ± 0.028</td>
<td>0.552 ± 0.050</td>
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<tr>
<td>7</td>
<td>P450 aromatase</td>
<td>0.728 ± 0.035</td>
<td>1.821 ± 0.047</td>
<td>0.791 ± 0.052</td>
<td>0.781 ± 0.028</td>
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*p<0.05, **p<0.01, ***p<0.001 compared to vehicle treated control.

*p<0.05, **p<0.01 compared to CC alone.

*p<0.05 compared to EB alone.
Fig. 10: Real time PCR of cyp19 (A,B,C) transcripts and corresponding (D,E,F) protein expressions as seen in Western blots (n=3) of testis, liver and brain preparations respectively. CC treatment effectively brought down the EB induced rise in cyp19 expression in the testis. *p<0.01, **p<0.001 compared to vehicle treated. #p<0.01 compared to CC alone. $p<0.05 compared to EB alone.

Table 3: Densitometric analysis of liver tissue after 15 and 30 days of treatment showing ratio of aromatase protein to β-actin expression ± SD.

<table>
<thead>
<tr>
<th>Treatment duration</th>
<th>Control</th>
<th>EB</th>
<th>CC</th>
<th>EB+CC</th>
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<tr>
<td>15 days</td>
<td>0.925±0.0515</td>
<td>0.850±0.034</td>
<td>0.873±0.0812</td>
<td>0.935±0.181</td>
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<tr>
<td>30 days</td>
<td>1.204±0.0408</td>
<td>0.653±0.029#</td>
<td>1.11±0.327</td>
<td>0.602±0.08**$</td>
</tr>
</tbody>
</table>

*p<0.01, **p<0.001 compared to vehicle treated control. 
#p<0.01 compared to CC alone. 
$ p<0.05 compared to EB alone.

Table 4: Densitometric analysis of brain tissue after 15 and 30 days of treatment showing ratio of aromatase protein to β-actin expression ± SD.

<table>
<thead>
<tr>
<th>Treatment duration</th>
<th>Control</th>
<th>EB</th>
<th>CC</th>
<th>EB+CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 days</td>
<td>0.855±0.026</td>
<td>0.707±0.019</td>
<td>0.652±0.042</td>
<td>0.601±0.591</td>
</tr>
<tr>
<td>30 days</td>
<td>0.767±0.10</td>
<td>0.606±0.08**#</td>
<td>0.749±0.0539</td>
<td>0.489±0.010**$</td>
</tr>
</tbody>
</table>

*p<0.01, **p<0.001 compared to vehicle treated control. 
#p<0.05, **p<0.01 compared to CC alone. 
$ p<0.05 compared to EB alone.
Chapter 5

To Investigate a Suitable and Appropriate Intervention to Counteract the Adverse Effects on Testicular Function Induced by Estrogen Administration