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Effect of Letrozole, a selective aromatase inhibitor, on testicular activities in adult mice: Both *in vivo* and *in vitro* study

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ABSTRACT

The aim of present study was to evaluate the significance of estradiol (E2) in testicular activities and to find out the mechanism by which E2 regulates spermatogenesis in mice. To achieve this, both *in vivo* and *in vitro* effect of Letrozole on testis of adult mice was investigated. Letrozole-induced changes in testicular histology, cell proliferation (proliferating cell nuclear antigen; PCNA), cell survival (B cell lymphoma factor-2; Bcl2), apoptotic (cysteine-aspartic proteases; caspase-3), steroidogenic (side chain cleavage; SCC, 3β-hydroxy steroid dehydrogenase enzyme; 3β HSD, steroidogenic acute regulatory protein; StAR, aromatase and luteinizing hormone receptor; LH-R) markers, glucose level, and rate of expression of glucose transporter (GLUT) 8 and insulin receptor (IR) proteins in the testis along with changes in serum E2 and testosterone (T) levels were evaluated. Letrozole acts on testis and caused significant decrease in E2 synthesis, but increase in testosterone level and showed regressive changes in the spermatogenesis. Letrozole-induced changes in various testicular markers were compared with the changes in serum E2 level. The correlation study showed that decreased circulating E2 level may be responsible for decreased insulin receptor (IR) level in the testis. The decreased effects of insulin inhibited the glucose transport in the testis by suppressing GLUT8. The decreased level of testicular glucose may produce less lactate as energy support to developing germ cells consequently resulting in decreased cell proliferation and cell survival, but increased apoptosis. Thus, Letrozole suppresses spermatogenesis by reducing insulin sensitivity and glucose transport in the testis, but significantly increased testosterone level by promoting gonadotrophin release by decreased E2.

1. Introduction

The widespread distribution of estrogen receptors and aromatase enzyme in male reproductive tract and impairment of male fertility due to lack of estrogen receptor (ER) alpha (Lubahn et al., 1993; Korach, 1994; Eddy et al., 1996) and aromatase (Toda et al., 2001; Robertson et al., 1999 and Honda et al., 1998) provided sufficient evidences reiterating the significance of estrogen (E2) as modulator of testicular activities. Aromatase, encoded by the CYP19A1 gene, is the key enzyme responsible for the synthesis of E2 from androgen. The E2 exerts its physiological effects via ER, α and β, localized throughout the male genital tract (Carreau et al., 2008). The role of E2 in testis was further substantiated by analysis of changes in testicular activities in transgenic mice deficient in aromatase, ER α and ER β genes. These knockout mice showed lack of endogenous E2 and were initially fertile but later on showed severe impairment of spermatogenesis (Eddy et al., 1996; Robertson et al., 1999). Studies conducted on rodents and human clearly show that E2 regulates proliferation, apoptosis and differentiation of germ cells as well as it regulates spermiation, transport and motility of spermatozoa, and scrotal testicular descent (Meccariello et al., 2014). These studies suggest that E2 is critical for a successful reproduction and fertility in male (Meccariello et al., 2014), however, more research is required to unveil the specific role of E2 in modulation of various testicular activities.

To elucidate the consequences of E2 deficiency on male reproductive function, we utilized the Letrozole, a highly potent, selective, third generation non-steroidal aromatase inhibitor, to suppress the endogenous E2 level in the male mice. Letrozole inhibits aromatase enzyme activity by binding with the heme iron of the enzyme. Letrozole-induced lowering of E2 is associated with an increase in levels of LH and FSH by negative feedback with a concomitant increase in testosterone (Raven et al., 2006; T'Sjoen et al., 2005). Aromatase deficient men also showed impaired spermatogenesis and sperm motility (Rochira et al., 2005; Rochira and Carani, 2009). Men with excess aromatase activity, reflected by low circulating T and relatively increased E2 level, showed severely

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defective spermatogenesis. Administration of aromatase inhibitors has recently been propose as a potential therapeutic option in men with low serum testosterone level and in obesity-related male hypogonadism (Lapauw et al., 2009). Despite the utilization of aromatase inhibitor as a potential treatment for a number of male reproductive disorders (Lapauw et al., 2009), the mechanism by which it affects various testicular activities are not clearly studied so far.

E2 exerts positive regulation on insulin action. So, lower E2 level suppresses insulin action or insulin receptor (Gupte et al., 2015). Apart from well known effect of E2 on testis, deficiency of E2 also shows a close association with changes in insulin and altered blood glucose homeostasis (Godsland, 2005). E2 deficiency in human is associated with deterioration in glucose homeostasis and development of IR and E2 replacement restores insulin secretion (Godsland, 2005; Jarvis et al., 2013).Glucose is the most important fuel of mammalian cells and its uptake is crucial for cellular metabolism, survival and proliferation. Accordingly adequate amount of glucose is required in the testis to achieve normal reproductive function (Rato et al., 2012). Hyperglycemia has been associated with the impairment of various functions of testis leading to male infertility (Amaral et al., 2008). It has recently been shown that LH-induced increased testosterone synthesis in Leydig cells are associated with increased transport of glucose (Banerjee et al., 2014). Insulin induced increased expression of GLUT8 is involved in increased uptake of glucose by testis (Banerjee et al., 2014). Insulin also exerts important role in the various testicular functions (Rato et al., 2012). It is thus possible that the decreased E2-induced suppressed testicular activity may be due to decreased glucose transport in the testis.

Therefore, the primary aim of present study was to evaluate the action of Letrozole associated decline in E2 on various testicular activities in mice. Additional aim of this study was to evaluate, whether E2 associated suppression in testicular activities is mediated through changes in insulin sensitivity.

2. Material and methods

Animal studies were approved by the institutional animal ethics committee (No. F.Sc./IAEC/2014-15/222). Adult male Parkes strain mice (weighing 25–30 g) of 12–13 weeks were housed under standard laboratory conditions, and were provided with pelleted food (Amrut Laboratory Animal Feeds, Pune, India) and water *ad libitum*. The mice were kept under controlled temperature (24 ± 2 °C) and humidity and 12 h of light: dark cycle. Experimental animals were housed separately in polypropylene cages (450 mm × 270 m m × 150 mm) with dry rice husk as bedding material. Animals were monitored regularly throughout the treatment period. Animals were maintained in accordance with the guidelines of the Banaras Hindu University Animal Ethics Committee.

2.1. The in vivo study

Mice were randomly divided into three groups ($n = 10$ per group) for *in vivo* study. The Group 1 was control and received the vehicle (1% aqueous solution of carboxmethylcellulose according to Kafali et al., 2004). The Group 2 and 3 were experimental received low dose (Let1 = 1 mg/kg body weight) and high dose (Let2 = 2.5 mg/kg/body weight) of Letrozole through oral gavage, for 28 days. At the end of the experiment, animals were killed by decapitation under a mild dose of anesthetic ether and blood was collected. Serum was collected from blood and kept at −20 °C for further assay. Testis of one side of each animal was kept at −20 °C for immunoblot and the other side of the testis was fixed in Bouin's fluid for immunohistochemistry.

2.2. The in vitro study

The different doses of Letrozole (L1 = 200 and L2 = 400 nM/ml) were selected. Adult male mice ($n = 6$) were killed by decapitation under a mild dose of anesthetic ether. Their testis were quickly dissected out and cleaned of any adhered fat tissue in DMEM (Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 mg/ml streptomycin sulfate. The testis were cut into equal pieces (≈10 mg in weight) and cultured by the method as described previously (Banerjee et al., 2012). Culture medium was a mixture of DMEM (with sodium pyruvate and L-Glutamine) and Ham's F-12 (1:1; v: v) (Himedia) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1% BSA (Sigma). After initial incubation for 2 h at 37 °C, culture medium was discarded and testis (one slice per tube) were finally cultured in 1 ml medium in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 18 h at 37° with two different doses of Letrozole (L1 = 200 and L2 = 400 nM/ml). In the control group (Lc) testicular sections were only incubated in culture media without any treatment. Each treatment group was run in triplicate. Testis cultured under these conditions appears healthy and do not show any sign of necrosis. Testis slices were collected at the end of culture, washed several times with PBS, and stored at −20 °C for immunoblot study, and media were collected and stored at −20 °C for steroid assay.

2.3. Histology

Testis fixed in Bouin's solution were embedded in paraffin wax and serially sectioned. Every fifth serial section were used to avoid repetition and to reduce the number of slides for histomorphometric study. One set of slide was used for haematoxylin and eosin staining to examine the effect of drug on testicular histology whereas other set was used for immunohistochemistry. Stages of spermatogenesis in the testes of mouse had been identified according to the criteria of Russell et al., 1990. We used seminiferous tubules of stage VII- VIII for histological and immunohistochemical analysis because it is the most frequent stage of spermatogenesis, highly dependent on androgen (O'Donnell et al., 1994) and seminiferous tubules have maximum mean diameter in this stage of spermatogenic cycle (Sarkar et al., 2015). The epithelium height and diameter of seminiferous tubules of control and treated testis was measured with motic software using Nikon-E200.

2.4. Immunohistochemistry

Immunohistochemistry was performed according to the method described earlier (Roy and Krishna, 2013). In brief, testis embedded in paraffin were sectioned at thickness 6 μm. The sections were deparaffinized in xylene followed by hydration through graded alcohol. The sections were then treated with 3% of H_2O_2 in methanol for blocking endogenous peroxidases. The sections were incubated with blocking serum for 3 h, followed by incubation with the primary antibody (PCNA, 1:200) for 4 h at room temperature. The sections were then washed and incubated with the horseradish peroxidase tagged secondary antibody for 2.5 h at room temperature (Anjum et al., 2014). After incubation of secondary antibody, sections were washed, and incubated with the chromogen substrate (0.1% 3, 3′diaminobenzidine tetra hydrochloride in 0.5 M Tris 7.6 and 0.01% H_2O_2) for 10 min. After this sections were dehydrated and mounted with DPX. Slides were analyzed under a light microscope (Nikon, Tokyo, Japan) and photographed. PCNA positive cells were also counted in 60 seminiferous tubules of each group.

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