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### **Alteration of fertility parameters in hyperandrogenic rodent model during implantation window**

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#### **ABSTRACT**

Early pregnancy loss is caused by poor endometrial receptivity. Implantation window is a bidirectional signaling event between fertile embryo and receptive endometrium, where fertile blastocyst implanted in to endometrial lining. It is the decisive duration for persistence of pregnancy. Receptivity of endometrium depends on rhythmic correlation between circulatory steroid hormones and certain important enzyme activities. Hyperandrogenism attributed to abnormal *in utero* development of fetus. In present study fertility parameters were evaluated in letrozole induced hyperandrogenic rodent model. For the study, implanted site from letrozol induce rat were removed and analyze for physical as well as important biochemical parameters during implantation window. Results showed that treated rodent model testosterone level was increased while estrogen and progesterone level were decreased. Alteration in physical parameters includes elevated body weight, prolong implantation time and increase resorption site compare to untreated rodent model. Activities of enzyme like alkaline phosphatase, Cathepsin and matrix metalloproteinase were reduced. It indicates that less endometrial receptivity during implantation window leads to early pregnancy loss.

#### **KEY WORDS:**

Endometrium receptivity, Fertility parameters, Hyperandrogenic condition, Implantation window.

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## INTRODUCTION

Reproduction is the event of life needed for propagation of progeny. Successful pregnancy requires implantation of an embryo. Implantation occurs during a confined period defined as 'implantation window' during which endometrium becomes receptivity for active trophoblast. Receptivity of endometrium is under the influence of ovarian steroids<sup>1</sup>. Pregnancy progressed through placenta formation and organogenesis. It is very complex process mediated by specific biomolecules in each phase. Alteration in these events leads to pregnancy loss.

Polycystic ovaries, increased body weight, placental thrombosis, deficient endometrium and hormonal deformity are some of the potential factors for pregnancy loss<sup>2</sup>. High androgen level is common feature observed in all condition clinically named as Hyperandrogenemia. Prevalence of hyperandrogenic condition affecting female in their reproductive age is 10% to 15% worldwide<sup>3</sup>. Such females are at alarming risk of miscarriages during first trimester<sup>4</sup>. Hyperandrogenic environment attributes abnormal *in utero* development of fetus<sup>5</sup>. Abnormality includes non-receptive and thin endometrium which results in implantation failure during early gestation<sup>6</sup>. Therefore implantation window becomes decisive phase which controlled by steroid hormones, specific biomarker and certain enzymes. Estrogen and progesterone plays momentous role in pregnancy. Progesterone is required for implantation, decidualization, embryogenesis, placentation and maintenance of pregnancy. Endometrial tissues proliferation, neovascularization and blood flow is controlled by estradiol during early pregnancy. Estradiol production in the ovary is controlled by membrane bound enzyme 17 $\beta$ -HSD<sup>7</sup>. 3 $\beta$ -HSD together with 17 $\beta$ -HSD play major role in steroidogenesis.

During implantation, complex proteins and polysaccharides of endometrium degraded by Cathepsin-D and Alkaline phosphatase<sup>8</sup>. Cathepsin-D involved in trophoblast invasion in to maternal endometrium by autolytic activity of epithelial cell<sup>9</sup> and makes contact with the maternal blood supply<sup>10</sup>. Alkaline phosphatase (ALP) required for implantation, uterine receptivity, decidualization and defense against bacterial endotoxin in rodent model<sup>11</sup>. Alteration in these molecular markers result implantation failure. Hence it would be interesting to study implantation enzyme activity and steroid status in endometrium during implanation window in letrozole induced hyperandrogenic rodent model. In other word these biomarker alteration plays role as indices for pregnancy status.

## **EXPERIMENTAL SECTION**

### ***Animals***

Adult virgin female Charles foster female rats (2-3 months) with 200–220 g body weight and regular estrus cyclicity were used for the study. All animals were housed in cages maintained in controlled temperature of  $25 \pm 1^\circ\text{C}$  and 45.5% relative humidity, with photoperiod cycle of 12 h: 12 h (light and dark) with free access of standard pellet food (Sai Durga Feeds and Foods, Bangalore, India) and water. Each experiment was approved by the Institutional Animal Ethics Committee, according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. (Reg.no.938/PO/ReBi/S/06/CPCSEA date: 30th June 2015 and Proposal No: BC/10/2016 was administered by the M.S. University of Baroda.

### ***Chemicals***

Nicotinamide adenine dinucleotide reduced (NADH), Dehydroepiandrosterone (DHEA), Estradiol, Iodo Nitro Tetrazolium Chloride (INT), p-Nitrophenol (PNP), para-Nitrophenylphosphate (pNPP), Hemoglobin substrate, Tyrosine were purchased from Sisco research laboratories Ltd., Mumbai, India. ELISA Kits purchased from Dia Metra, Germany. All other chemicals like Letrozole, anhydrous Magnesium chloride, Sodium hydroxide, Potassium hydrogen phthalate, Tween -20, Glycine, Folin & Ciocalteu's (FC) phenol reagent, Acetic acid, Sodium acetate, Ethanol, and Trichloroacetic acid (TCA) were purchased locally.

### ***Development of Hyperandrogenic condition Rats***

Adult virgin female rats were treated with 0.5 mg/kg body weight of letrozole<sup>12</sup> which serve as test (hyper androgenic condition) while control group received 1% carboxymethylcellulose (CMC) orally for 21 days. After treatment, these both groups were validated for hyperandrogenic condition by monitoring estrus cyclicity, steroid hormone level and body weight. Further these animals were kept for mating with fertile adult male rats. Presence of sperms in vaginal smear early in morning was considered as Day 0 of pregnancy. All the parameters were evaluated in the early gestation period (5<sup>th</sup>-8<sup>th</sup> Day). The steroid hormone (progesterone, estradiol and testosterone) status were evaluated in the serum by ELISA. The animals were sacrificed. Steroid enzyme (3 $\beta$  HSD & 17 $\beta$  HSD) and implantation enzyme (Cathepsin and Alkaline Phosphatase) activities were evaluated in the implanted region of the uterus.

### ***Hormonal profile***

Blood samples were collected in fresh vials (without any anticoagulants) and centrifuged at 1200 g for 15 minutes to separate serum for hormonal profile by using ELISA kits procured from Diametra Inc, Germany. Samples were processed according to Kit (Meril Diagnostics, Canada) protocol. Briefly, serum was added [For Progesterone 20µl, Testosterone 25 µl, Estradiol 25 µl] in pre-coated ELISA plate. The plate was incubated for 1 hour. After incubation, the plate was washed 3 times with 300 µl wash buffer and added 100µl of TMB (3,3',5,5'-Tetra methyl benzidine) substrate with 15 min incubation in dark. Then 100µl of Stop solution was added to each well to stop the reaction. Shake gently and read at 450 nm.

### ***3 beta- and 17 beta-hydroxy steroid dehydrogenase***

10% implanted uterine homogenate was prepared in 0.1 M Tris HCl buffer (pH 7.8) and centrifuged at 10,000 rpm for 30 min at 4°C. Supernatant was used to checked enzyme activity. 0.1 M Tris-HCl buffer (pH 7.8) containing NAD (500µM) and the substrate DHEA (100µM) for 3 β HSD and estradiol (100µM) for 17 β HSD were taken and total volume of 3 ml was make up using distilled water. The reaction started by adding the 100 µl supernatant (enzyme) and 100µl color reagent INT followed by incubation at 37°C for 1hr. The reaction terminated by the addition of 2.0 ml of phthalate buffer (pH3.0) and read at 490 nm. The enzyme activity was calculated from the standard curve of NADH and expressed as nmoles NADH formed /min/1mg protein<sup>13</sup>

### ***Alkaline phosphatase (ALP)***

10 % implanted uterine homogenate was made in glycine buffer centrifuged at 900 g for 10 min. Equal volume of glycine and PNPP was used as working substrate. 25 µl homogenate was added in 500 µl working substrate and incubated at 37°C for 30 min. The reaction terminated by the addition of 5ml of 0.05 N NaOH and read at 405 nm. The enzyme activity was calculated from the standard curve of PNP and expressed as µmoles PNP formed /min/1mg protein.<sup>14</sup>

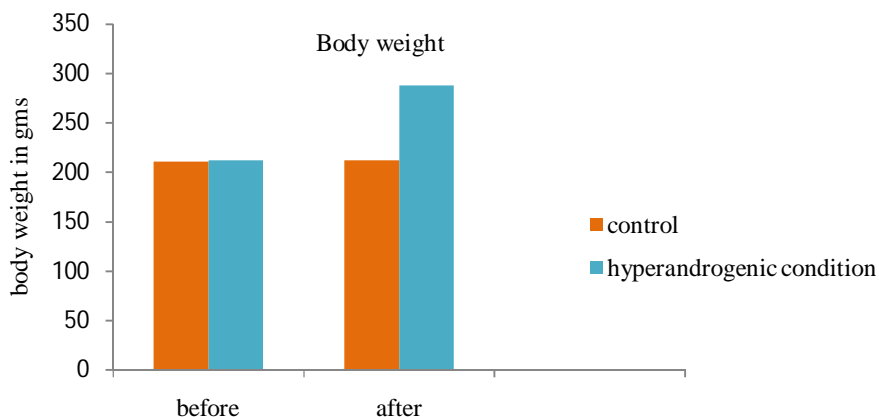
### ***Cathepsin- D***

Implanted site from uterus was excised and 10 % homogenate was made in Acetate buffer and centrifuged at 10,200 g for 30 min. 200µl of supernatant was added in 100µl of hemoglobin substrate shake vigorously and keep at 37°C for 10 min. Precipitates were obtained by adding 2ml 0.3 M TCA with vortex for 1 min and kept at room temperature for 30 min. Precipitates were separated by filtering through whatman filter paper. 1 ml of filtrate was added 2 ml 0.5 N NaOH and 600 µlFC reagent. After 5 min incubation absorbance was taken at 750 nm. The enzyme activity was

calculated from the standard curve of tyrosine and expressed as  $\mu\text{moles tyrosine formed /min/mg protein}^{15}$ .

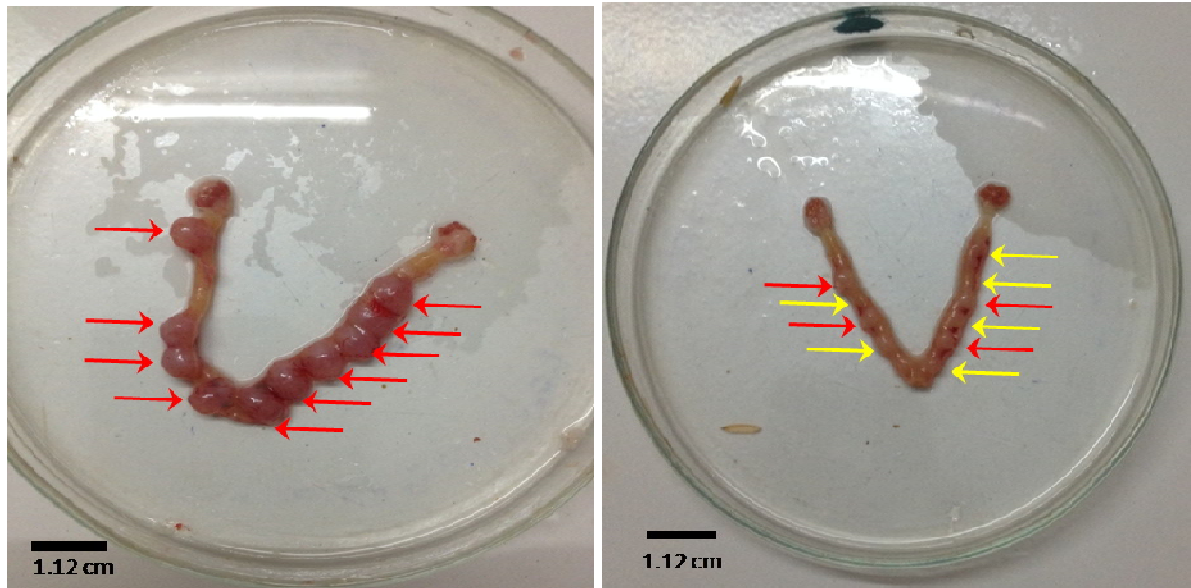
## RESULTS

Adult female Charles foster rats (200-210 gm) showing normal estrus cyclicity were injected with Letrozole for 21 days orally to induce hyperandrogenic condition. 0.5 mg/ body weight letrozol increased testosterone level form  $0.41 \pm 0.08 \text{ ng/ml}$  to  $1.13 \pm 0.15 \text{ ng/ml}$  (Data not shown)<sup>16</sup>. After 21days treatment bodyweight of hyperandrogenic rats were increase to 270-280 gm compare to normal (Figure-1). Results are similar to that reported in PCOS women with high BMI ( $80.9 \pm 11.3 \text{ kg}$ ) and low BMI ( $62.4 \pm 6.2 \text{ kg}$ ) compare to the normal (For high BMI  $79.1 \pm 15.6 \text{ kg}$  and for low BMI  $59.1 \pm 5.6 \text{ kg}$ ) (Moran et al, 2015). BMI of PCOS women ( $33.5 \pm 7.6 \text{ kg/m}^2$ ) was higher than BMI of normal women ( $25.6 \pm 5.9 \text{ kg/m}^2$ )<sup>17</sup>.



**Figure-1 Difference in body weight before and after the treatment with letrozole**

Four implanted site and six resorb sites were present in hyperandrogenic rat model compare to normal rat model which showed 10 implanted sites but no resorb site in uterus at their implantation window (Figure-2). No implanted sites were reported in PCOS rat model induced by dihydroxyepiandrosterone after five days of implantation<sup>18</sup>. Prolonged conceptive time i.e. seven cycles was observed in treated rat. Untreated rate conceptive time was 3 cycles. Estradiol level was decreased from  $71.3 \pm 9.4 \text{ pg/ml}$  to  $34.6 \pm 3.5 \text{ pg/ml}$  while progesterone level was also decreased from  $50.0 \pm 6.3 \text{ ng/ml}$  to  $11.8 \pm 1.6 \text{ ng/ml}$  (Detail data not shown)<sup>16</sup>.



**Figure-2 Implanted uterus from Normal (Left) and hyperandrogenic (right) rat model  
Red Arrow– Implanted site; Yellow Arrow– Resorb site**

Steroid enzyme  $3\beta$  HSD activity was analyzed spectrophotometrically for implanted uterus on fifth, sixth, seventh and eighth day. The activity was highest at day sixth ( $0.70 \pm 0.04$  nM of NAD formed/min/ng of protein) compare to the normal rodent model ( $1.01 \pm 0.02$  nM of NAD formed/min/ng of protein) (Figure-3). In case of PCOS women  $3\beta$  HSD activity were reported to increase in placenta region<sup>19</sup>. Activity of  $17\beta$  HSD was decrease form  $0.74 \pm 0.03$  nM of NAD formed/min/ng of protein to  $0.55 \pm 0.02$  nM of NAD formed/min/ng of protein (Figure-4).  $3\beta$  HSD and  $17\beta$  HSD both were reported to be decrease in PCOS insulin resistant patient which interfere in insulin signaling and steroidogenesis<sup>20</sup>. Similarly these enzymes found less active in hyperandrogenic female rate injected with equine Chorionic gonadotropin<sup>21</sup>. Cathapsin-D activity was decrease from  $27.40 \pm 2.56$  nM of tyrosine formed/min/ng of protein to  $22.85 \pm 1.44$  nM of tyrosine formed/min/ng of protein on sixth day of implantation (Figure-5). The results are supported by the results documented in rat model during atresia. Ovaries treated with PMSG reported decrease in cathapsin activity of granulosa cells<sup>22</sup>.

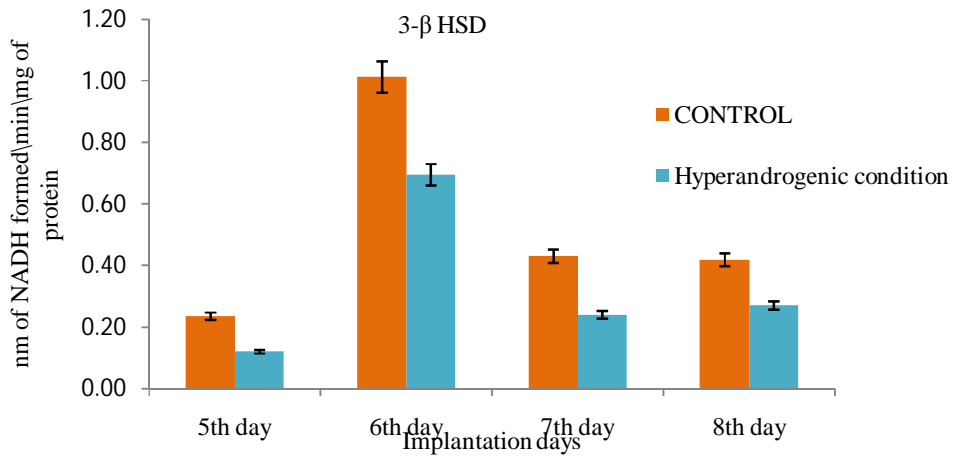


Figure-3 Activity of 3-β HSD in normal and hyperandrogenic rodent model

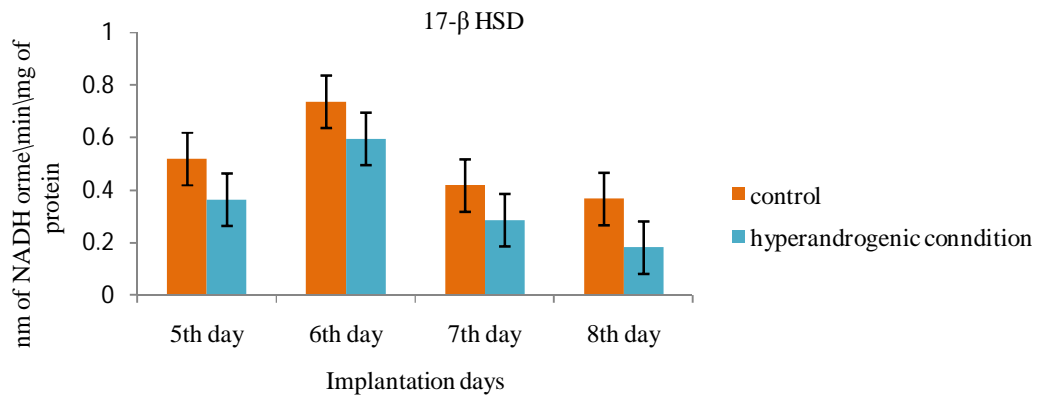


Figure-4 Activity of 17-β HSD in normal and hyperandrogenic rodent model

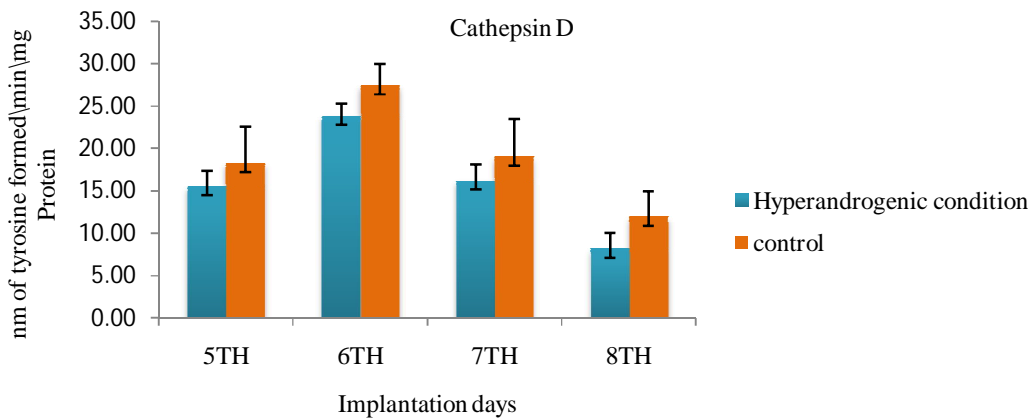


Figure-5 Activity of Cathepsin-D in normal and hyperandrogenic rodent model

Activity of ALP was decreased on seven. Normal rodent model showed  $14.84 \pm 0.24 \mu\text{M}$  of PNP formed/min/mg while hyperandrogenic rodent mode showed  $9.91 \pm 1.63 \mu\text{M}$  of PNP formed/min/mg (Figure-6).

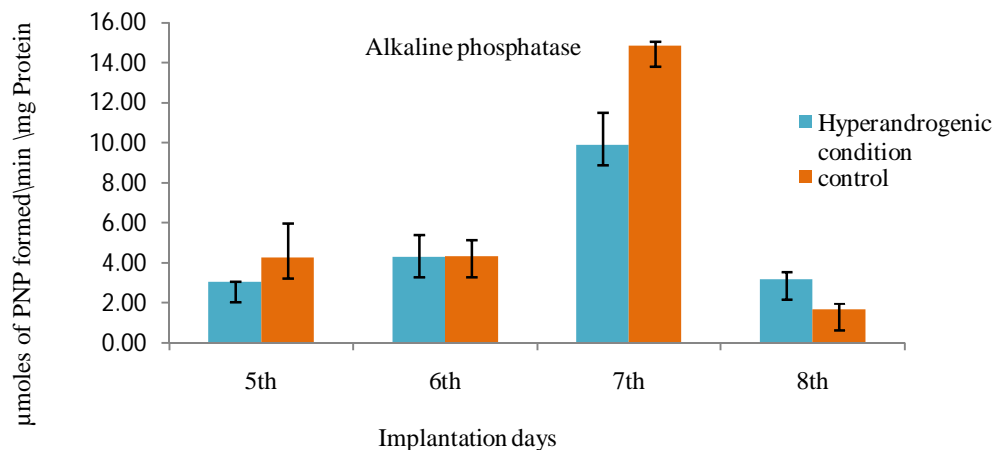


Figure-6 Activity of ALP in normal and hyperandrogenic rodent model

## CONCLUSION

Current study principally emphasized that letrozole induce Hyper androgenic rat model showed increase in body weight with arrested estrus cycle, increased conception time(7 weeks) as compare to the normal(3 weeks). Hyper androgenic condition also affects fertility parameters by decreasing activity of  $3\text{-}\beta$  HSD,  $17\text{-}\beta$ HSD, ALP and Cathepsin-D. These indicate poor endometrial receptivity.

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