Uterine Implantations and Litter Size Alteration in Anti-inhibin and eCG-hCG Treated Virgin Pregnant Female Rats

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Abstract

To investigate the comparative roles of anti-inhibin and eCG-hCG treatment on uterine implantation sites and litter size in virgin female rats, 120 virgin cycling female rats, aged 56 days and weighted 150-170 g., were randomly assigned to three equal groups. At late metaestrus and 54h later, the females were supplemented with intraperitoneal injection of 1µg anti-inhibin and 100µl normal saline, respectively (AI group), 20IU eCG and 10IU hCG, respectively (eCG group), and 100µl twice, respectively (Control). After mating, blood samples were obtained daily from 6 females of each group, at the first 4 days, for hormonal assay (FSH, LH, 17B-estradiol, and prolactin). At day 5 of pregnancy, 6 females from each group, were injected with 1% (w/v) pontamine blue (500 µl, iv). After 15 min., the females were sacrificed for counting of uterine implantations. The remaining 18 females continued to the end of gestation. Litter numbers were counted. Serum FSH in AI females registered higher levels than others throughout the four days of pregnancy, whereas LH levels showed no differences, except day one which recorded significant elevation in eCG group. Prolactin levels increased significantly in AI group throughout the four days of pregnancy. Estradiol concentrations showed insignificant differences among groups except day one which showed significant elevation in AI group. Uterine implantation sites increased significantly in AI group compared with others, also, eCG group recorded higher number than control. In conclusion treatment of virgin female rats with anti-inhibin, at late metastrus, increases the reproductive efficiency and multiple births.

KEYWORDS: Anti-inhibin, eCG, fertility, Implantation sites, Litter size

Introduction

Production of gonadotropic hormones (LH and FSH) is differ among estrus phases, as the positive feedback of estrogen hormone throughout the follicular phase lets a surge of LH and FSH release into the circulatory system to reach ovary and stimulates ovulation (Guthrie *et al.,* 1992). FSH is produced inside pituitary gonadotrophs and is an important component of the reproductive activities. FSH has a numerous of activities in female reproductive organs including proliferation and differentiation of granulosa cells in ovaries. FSH has direct actions in the production of female gametes, in addition to hormonal secretion (such as estradiol and inhibins) that performs feedback action on FSH secretion from the anterior pituitary (Rozell and Okrainetz, 2009).

Inhibins are biosynthesized by ovarian follicles by means of small antral development to preovulatory stages (Medan *et a*l, 2007). Activins and inhibins are the reproductive influencing members of the transforming growth factor β superfamily. The

antagonistic relationship between activins and inhibins is so important in the integration of reproductive functions. While activin stimulate follicle-stimulating hormone (FSH) secretion from the adenohypophysis, the important role of inhibin as feedback regulator of FSH secretion from adenohypophysis and therefore in ovarian folliculogenesis is well recognized by several researchers (Anuradha *et al*, 2012). FSH is known to be the key inducer hormone for growth and development of ovarian follicles. The rate of FSH concentrations decrease is directly correlated with the reduction of growing cohort follicles number. Therefore, secretion of inhibins from the growing cohort follicles into the circulation is probably highly related with the decline of FSH secretion leading to atresia (Medan *et al*, 2007).

The results of various trials confirmed the participation of inhibin, in female reproduction, as a regulator of FSH secretion throughout the growth phase of the dominant follicle(s) in the early luteal phase, where it has been found a hypersecretion of FSH after neutralization of endogenous inhibin throughout the early luteal phase, which associated with stimulation of follicular growth and development. These findings indicated the role of inhibin as an impact negative regulator of FSH secretion throughout the early luteal phase which is normally associated with the high secretion of estradiol-17β and progesterone (Hillier, 1991). By mid follicular phase of estrus cycle, the activity of granulosa cell aromatase, the production of inhibin and the expression of LH receptor showed high elevation to critical levels. This coupling between aromatase activity increment, inhibin production and LH receptors sensitization leads to selection of the destined follicle for ovulation (Hillier, 1991).

The combination of eCG and hCG has been used as a common method for induction of superovulation in different farm animals (Rahman *et at*., 2008; Moeini *et al*, 2009), whereas passive immunization against endogenous inhibin-α subunit improved the ovulation rate via elevated FSH secretion in rats (Rivier and Vale, 1989; Al-Sa'aidi and Samir, 2010), mice (Medan *et al.*, 2004), guinea pigs (Shi *et al.*, 2000), hamsters (Kishi *et al.*, 1997), ewes (Maan *et al.*, 1993), cows (Takedomi *et al.*, 1997), goats (Medan *et al.*, 2004), and mares (Nambo *et al.*, 1998). The present experiment was conducted to evaluate the the role of passive immunization against inhibin alpha subunit and eCG-hCG treatment at late metaestrus on uterine implantation sites and litter size.

Materials and methods

Animals: Adult virgin cycling female Wistar rats, aged 56 days; and weighted 150-165 g., have been used in the present study. They were kept under controlled day light (12L: 12D cycles) and temperature (22-24 °C) with access to standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water *ad libitum*. Vaginal smears have been checked daily and only female rats with at least two consecutive 4-5 day cycles have been used.

Experimental design: one hundred and twenty female rats have been assigned into three equal groups (AI, eCG, and control). AI group (40 female rats) have been administered a single ip injection of anti-inhibin (1 µg/ rat), dissolved in 100 µl of distilled water, at late metaestrus. After 54 h (at proestrus), females have been injected with normal saline (100µl/ rat). eCG group (40 female rats) have been administered with ip injection of eCG (20 IU/ rat), dissolved in 100 µl of distilled water, at late metaestrus. After 54 h (at proestrus), females have been injected with 10 IU hCG. Control group (40 female rats) have been administered with ip injection of normal saline (100µl/ rat), at late metaestrus and after 54 h (at proestrus). To study the effect of treatment with anti-inhibin and eCG on fertilization and the number of implantation sites, the remained females have been mated with experienced fertile males on the

evening of proestrus (1 male: 3 females). To ensure pregnancy, the presence of vaginal plug, in the next day of mating, has been examined. This day was denoted as zero day of pregnancy. Starting from day one until day four of pregnancy, 18 females (6 from each group) have been sacrificed each day. Blood samples were obtained and sera were separated and stored at -22C until hormonal assay (FSH, LH, estradiol 17B, and prolactin) using ELISA technique. Anti-inhibin alpha that used in the present study was obtained as purified antibody (monoclonal antibody) at a concentration of 200 µg/ ml. At day 5 of pregnancy, 18 females (6 from each group), have been administered with iv injected of a 1% (w/v) pontamine sky blue solution (500 µl) under slight ether anesthesia on day 5 of pregnancy, to extravasation of the stain into the implantation area in the endometrium, then the females have been sacrificed after 15 minutes of administration and implantation sites have been counted. Ten pregnant females have been allowed to continue until delivery. Litter number for each female has been recorded.

ELISA technique for hormonal assay in serum: Depending on the manufacturer instructions (ABO, Switzerland), serum insulin concentration has been estimated.

Statistical analysis: Results were expressed as mean ± standard deviation. Comparisons between groups and periods values were performed using one way analysis of variance (ANOVA1) and newman- keuls. Differences were considered to be significant at the level of $P<0.05$. Statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA).

Results

Hormonal profile:

FSH: figure (1) revealed significant ($p\leq0.05$) higher level of serum FSH concentration in AI treated female rats than eCG treated or control females throughout the first four days of gestation, whereas control group recorded significant ($p\leq0.05$) higher concentration compared with that of eCG treated group during 1st, 2nd, and 3rd days of gestation, but they recorded no significant ($p\geq0.05$) difference at the 4th day of gestation. In comparison between periods for each group, the concentration of all groups showed significant decline ($p\leq0.05$) as gestation period progressed.

LH: The results of serum luteinizing hormone (LH) concentration (ng/L) in virgin pregnant female rats during the first four days of pregnancy, clarified in figure (2), showed no significant ($p\geq0.05$) differences among the experimental groups at 2nd, 3rd, and 4th days of pregnancy, whereas 1st day registered significant ($p\leq0.05$) elevation in eCG treated pregnant rats in comparison with that of other groups. In comparison among periods for each group, the concentration in AI and control groups recorded no significant ($p\geq0.05$) differences in comparison between 1st, 2nd, and 3rd days of pregnancy, but they were significantly ($p\leq0.05$) higher than that of 4th day of pregnancy. On the other hand, the concentration of eCG treated group at 2nd and 3rd days of pregnancy showed no significant ($p\geq0.05$) difference between each other but they were significantly ($p\leq0.05$) higher than that of 4th day of pregnancy, and significantly ($p\leq0.05$) lower than that of 1st day of pregnancy.

Prolactin: serum concentrations of prolactin (ng/L) in virgin pregnant female rats, clarified in figure (3), revealed significant ($p\leq0.01$) higher concentrations in AI group at all periods in comparison with the corresponding levels of eCG treated and control females, which showed no significant ($p\geq0.05$) differences when compared with each other. In comparison between periods, in AI group, the concentration registered significant ($p\leq0.05$) gradual decline with time, whereas that of eCG treated and control groups showed no significant ($p\geq0.05$) differences between 1st and 2nd days of

pregnancy, then they recorded significant ($p \leq 0.05$) gradual decrease as the gestation progressed.

Estradiol 17B: The results of serum estradiol 17B concentrations in pregnant female rats, clarified in figure (4), showed increased ($p \leq 0.05$) level of AI treated group at 1st day of pregnancy than eCG treated and control groups, which showed no significantly ($p \geq 0.05$) difference between each other, whereas 2nd, 3rd, and 4th days of pregnancy showed no significant ($p \geq 0.05$) differences between experimental groups. In comparison among periods, all groups at 1st day of pregnancy recorded significant ($p \leq 0.05$) higher levels of serum estradiol concentrations compared with 2nd, 3rd, and 4th days of pregnancy, whereas the last three days recorded no significant ($p \geq 0.05$) differences when compared with each other.

Uteri implantation sites:

The results of uteri implantation site, clarified in figures (5) and (6), showed elevated ($p \leq 0.05$) number of AI treated pregnant female rats (10.50±1.04) compared with that recorded by eCG treated (6.50±1.04) and control (3.50±1.04) female rats. On the other hand, statistical analysis showed significant ($p \leq 0.05$) difference in the implantation sites numbers between eCG treated and control virgin female rats.

Litter size: figure (7) revealed that treatment with anti-inhibin increased the delivered litter number of virgin female rats (10.66±1.50) compared with females of eCG treated (7.00±1.00) or control (2.66±1.15).

Discussion:

In previous studies, it has been reported that the most available method for induction of superovulation is treatment with a combination of eCG and hCG. However, numerous studies have reported high rates of pre- and post-implantation losses in animals that treated with eCG, where this failure has been shown to be related to a maternal environment (Leveille and Armistrong, 1989), which may due to excessive follicular stimulation related to the long half-life of eCG. High doses of eCG may have a detrimental effect on oocyte quality, such as the ability to be fertilized (Walton *et al*., 1983; Evans and Armstrong, 1984), impaired development (Miller and Armstrong, 1981; Ertzeid *et al*., 1993), and/or chromosomal abnormalities (Yun *et al*., 1989). Therefore, in the present study, we tried to find an alternative method for producing large number of viable oocytes and zygotes in virgin female rats.

Inhibin is an essential regulatory hormone with potent bioactivity for inhibiting FSH secretion from adenohypophesis and therefore regulating FSH secretion in various mammals (de Jong 1988). In previous studies, a negative relationship between plasma concentration of FSH and inhibin has been established (Taya, 1993; Araki *et al* 2000). Multiple ovulations have been induced successfully by passive immunization against endogenous inhibin in female rats (Rivier and Vale, 1989; Al-Sa'aidi and Samir, 2010). Furthermore, many studies have reported that oocytes superovulated with immunization against endogenous inhibin have the ability to develop normally (Takedomi *et al*., 1997; Shi *et al*., 2000; Wang *et al*., 2001).

The decreased serum inhibin concentration due to immunoneutralization of endogenous inhibin could accompany by increased serum activin and FSH concentrations (Abdulla, 2013; Thanoon, 2013; Al-Sa'aidi *et al*., 2013). Therefore activin-A could perform its action on pituitary gonadotrophs to secrete more FSH because inhibins and activins are functionally antagonistic members of the evolutionarily conserved TGFb family of extracellular signaling molecules (Miyazono *et al.*, 2010). Increased FSH concentration along with increased estradiol-17β is perhaps the main reason for the improved reproductive efficiency seen in the current

study which may potentially reflected on pituitary gland functions (Miyazono *et al.*, 2010).

In comparison with eCG treated and control female rats, serum concentration of estradiol-17β in the anti-inhibin treated female rats increased significantly which occurred in concomitant with the notable growth of a large number of ovarian follicles in anti-inhibin treated females and those treated with eCG compared with control group, as it has been found that passive immunization of female rats against inhibin alpha subunit increased folliculogenesis as well as Graffian and total follicle number (Al-Sa'aidi and Samir, 2010). These results clearly indicate that passive immunization against inhibin alpha subunit enhances biosynthesis of estrogens from ovarian follicles. These findings suggest that a high level of endogenous FSH stimulates the wave of follicular development and results in production of a large amount of estradiol-17β, which induces the LH surge by positive feedback effect to the hypothalamus and pituitary axis, leading to induction of superovulation.

In the present study, the resulted gradual increase of serum prolactin concentration and gradual decrease of serum estradiol-17β and FSH concentration as gestation progressed could be attributed to the feedback role of estradiol on hypothalamus. Several studies support the hypothesis that hypothalamic AT1 receptors participate in the ovarian steroid feedback on prolactin secretion. The number of AT1 receptors in the arcuate nucleus is inversely related to prolactin secretion, which they are low during proestrus (low estradiol concentration) and highest at estrus (high estradiol concentration) and confined to the dorsomedial portion of the arcuate nucleus where the cell bodies of the TIDA system are located (Warembourg *et al* 1989). It has been reported that prolactin level increase gradually during pregnancy and reach their highest value at last trimester of gestation (Kandiel *et al*., 2010). Moreover, they provided evidence that central PRL signaling provides more inhibition of the LH surge that coincide with the generation of the PRL surge. These observations assist the interpretation of previous reports on the role of PRL in modulating hypothalamic circuits important in regulating mammalian reproduction (Grachev *et al* 2010).

The results of the present study clearly showed that adult rats can be effectively superovulated to produce more than a threefold increase in ovulation rate over control animals and a twofold increase over eCG treated virgin female rats through passive immunization against inhibin alpha subunit. Only a single injection of inhibin alpha antiserum is needed to induce superovulation in adult rats, making it more practical and efficient compared with the previous studies, such as eCG-hCG (Welschen and Rutte, 1971; Mukumoto *et al*., 1995), FSH preparations (Hamilton and Armstrong, 1991), and recombinant human FSH (van Cappellen *et al*., 1997).

Immunization against inhibin enhances FSH secretion and follicular growth and finally increases the ovulation rate. The different studies on inhibin for increasing ovulation rate and fecundity in animals opens the possibility of eliminating the use of exogenous gonadotropins for fertility enhancement (Bhardwaj *et al*., 2012). On the other hand, the present hormonal changes and high number of delivered litters indicates a highly significant potency of immunonutralization against inhibin alpha subunit in virgin cycling female rats. This elucidation, of its action, may lead to the development of new strategies aimed at supporting reproduction and its future performance, therefore, passive immunization against inhibin alpha subunit may play a significant potent role in animal reproduction applications.

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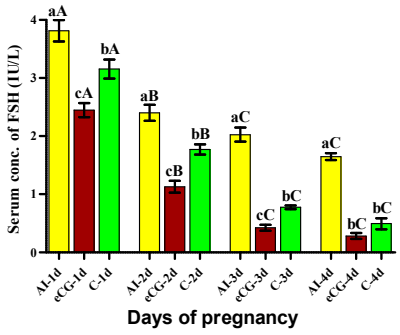


Figure (1): Serum FSH concentrations (IU/L) in AI and eCG treated pregnant female rats.
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.

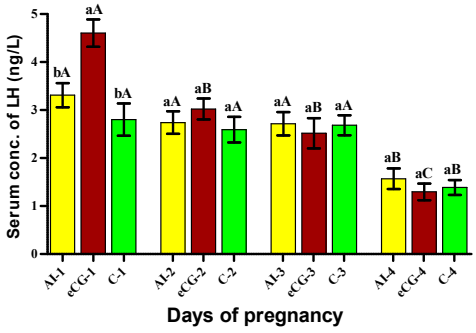


Figure (2): Serum LH concentrations (ng/L) in AI and eCG treated pregnant female rats.
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were

presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.

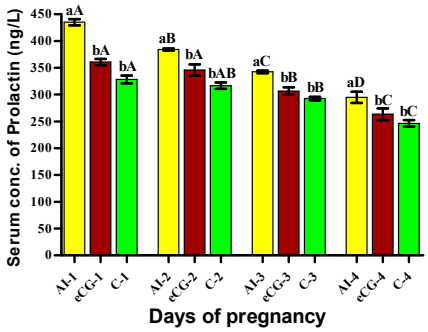


Figure (3): Serum prolactin concentrations (ng/L) in AI and eCG treated pregnant female rats.

AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.

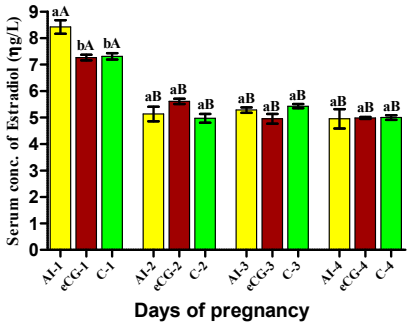


Figure (4): Serum estradiol 17B concentrations (ng/L) in AI and eCG treated pregnant female rats.

AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.

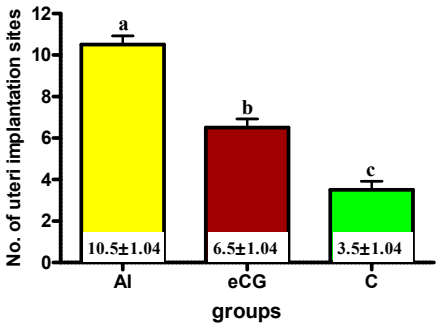
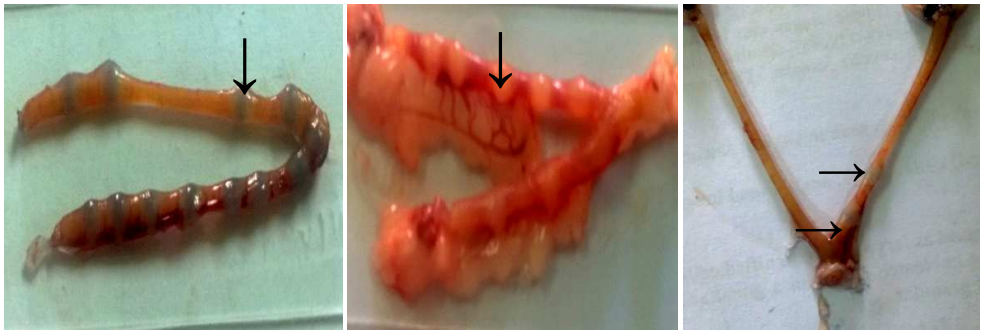


Figure (5): Uterine implantation sites in AI and eCG treated pregnant female rats.
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.



Group AI **Group eCG** **Group C**

Figure (6): Uterine implantation sites in AI and eCG treated pregnant female rats. This figure shows uteri stained with Pontamine blue (1%).
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.

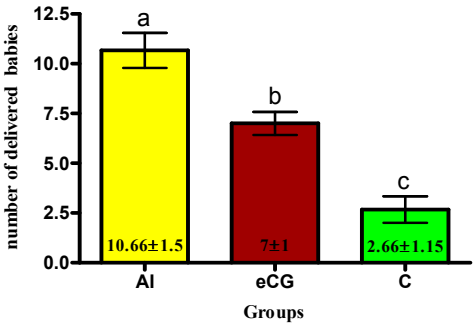


Figure (7): delivered litter size in AI and eCG treated pregnant female rats.
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100 µl of dw /rat *ip*, after 54 h. C: female rats injected with 100 µl of dw /rat *ip*, in late metestrus and after 54 h. Data were presented as Mean ±SD of 6 observations (n=6). Different small litters denote significant difference ($p \leq 0.05$) between groups for each period. Different capital letters denote significant difference ($p \leq 0.05$) between periods for each group.