*Comparative effect of anti-inhibin and eCG-hCG supplementation on reproductive hormonal profile in virgin cycling female rats.

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Abstract
To examine the effect of anti-inhibin and hCG-eCG treatments on reproductive hormonal profile in virgin cycling female rats, fifty four virgin cyclic female rats, aged 56 days and weighted 150-170 g., were randomly assigned to three equal groups (AI, eCG, and control). At late metaestrus and 54 h later, AI females were injected with anti-inhibin (1 µg/rat, ip) and normal saline (100µl/rat, ip), eCG females were injected with equine chorionic gonadotropin (20 IU/rat, ip) and human chorionic gonadotropin (10 IU/rat, ip), whereas control females were injected with normal saline (100µl/rat, ip). Eighteen females from each group were sacrificed after treatment (6 females/ group every 12 h for three times). Blood samples were obtained for hormonal assay (FSH, LH, estradiol 17B, prolactin, and ir-inhibin B). Serum FSH concentration of AI females increased significantly than other other groups throughout all periods. Also control females recorded significant elevation compared with eCG females. Serum LH concentrations registered no significant differences between AI and C groups, but they were significantly higher than eCG group only after 36 h. of treatment. Serum prolactin concentrations showed significant decline in AI group among experimental groups throughout all periods. Inhibin B levels, after 12, 24, and 36 h of treatment, decreased significantly in AI group compared with other groups. 17B-estradiol concentrations recorded insignificant differences among groups after 12 h. of treatment, while after 24 h., AI group level increased significantly than other groups. After 36 h. of treatment, AI and eCG groups recorded insignificant difference between each other, but, they were significantly higher than control. It can be concluded that anti-inhibin treatment increases the reproductive fecundity in female rats.
Key words: anti-inhibin, eCG, fertility, reproductive hormones.

Introduction
Inhibin, a glycoprotein hormone produced by the granulosa cells in the ovarian follicles suppresses the production and secretion of pituitary gonadotropins, particularly FSH through negative feedback mechanism, as well as its participation in the regulation of ovarian folliculogenesis through autocrine and/or paracrine control (1). Passive immunization against inhibin raised FSH secretion in the phase of the estrous cycle with high estradiol secretion (follicular phase) (2) or with high progesterone secretion (mid-luteal phase) (3). Multiple ovulations were encouraged effectively by endogenous inhibin immunoneutralization in a number of species such as mice (4), rats (5), hamsters (6), cows (7), and mares (8). Early onset of puberty (time of first ovulation) was observed after immunization early with an inhibin-enriched bovine follicular fluid preparation (9). Immunoneutralization against endogenous inhibin has been used to enhance oocyte development (10), and accelerate puberty in immature female rats (5,11). The present study aimed to examine the role of passive immunization against inhibin alpha subunit and eCG-hCG treatment at late metaestrus on reproductive hormonal profile in virgin cycling female rats.

Materials and methods
Animals: Adult virgin cycling female rats of Wistar strain (aged 56 days; and weighted 150-170 g.) have been born at the animal house of the College of Veterinary Medicine, Al-Qadisiya University, and 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*. The females were identified by tail labeling.

Vaginal smears have been checked daily and only female rats with at least two consecutive 4-5 day cycles have been used.

Experimental design: fifty four female rats have been assigned into three equal groups (AI, eCG, and control). AI group (18 females) have been administered a single ip injection of inhibin antiserum (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 (18 females) 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 (18 females) have been administered with ip injection of normal saline (100µl/ rat), at late metaestrus and after 54 h (at proestrus). After 12, 24, and 36 hours (during estrus cycle), 6 females from each group have been sacrificed each time, and blood samples have been obtained in non-heparinized centrifuge tubes. Blood sera were separated and stored at -20 ºC until hormonal assay. Hormonal concentrations (FSH, LH, estradiol 17B, prolactin, and ir-inhibin B) were assessed 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.

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

FSH: serum FSH (IU/L) increased significant ($p\leq0.05$) after 12 h of AI treatment compared with other groups, and continued after 24 and 36 h of treatment (figure 1), whereas eCG treated group showed significant decline ($p\leq0.05$) compared with control throughout the three experimental periods. In comparison between periods for each group, the differences reached its peak after 36 h of treatment in all groups.

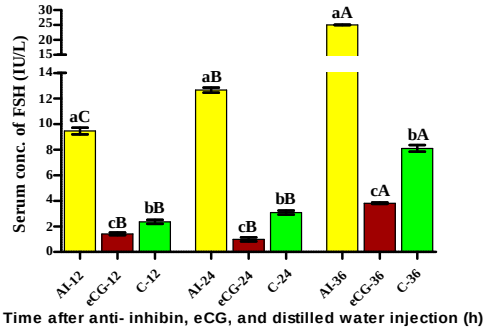

Figure (1): Serum FSH concentrations (IU/L) in AI and eCG treated female rats.

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 ($p\leq0.05$) between groups for each period. Different capital letters denote significant difference ($p\leq0.05$) between periods for each group.

LH: the results of serum LH concentration (ng/L) in virgin cyclic female rats showed no significant ($p\geq0.05$) differences between groups after 12 and 24 h of treatment, when compared with each other. After 36 h of treatment, AI treated and control females recorded no significant ($p\geq0.05$) difference, but they were significantly ($p\leq0.05$) higher than that registered by eCG treated females. In comparison between the experimental periods (12, 24, or 36 hrs. after treatment) for each group, the statistical analysis showed that all groups recorded the significant ($p\leq0.05$) highest levels after 36 h of treatment in comparison with that recorded after 12 and 24 h of treatment, which showed no significant ($p\geq0.05$) differences when compared with each other (figure 2).

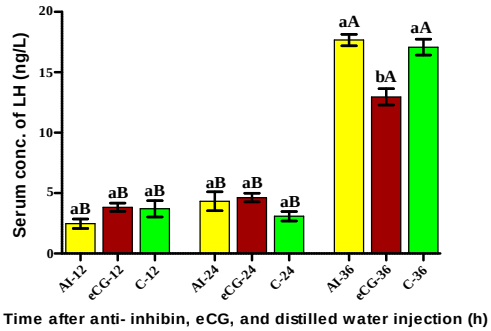

Figure (2): **Serum LH concentrations (ng/L) in AI and eCG treated female rats.**
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU of hCG/100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

Prolactin: serum concentrations of prolactin (ng/L) in virgin cyclic female rats, clarified in figure (3), revealed significant ($p \leq 0.01$) lower concentrations in AI treated females compared among experimental groups at all periods (12, 24, and 36 h after treatment), whereas eCG treated and control females recorded no significantly ($p \geq 0.05$) differences in their serum prolactin concentrations at the same periods. Statistical analysis among periods, for AI treated group, showed significant ($p \leq 0.05$) decline of serum prolactin concentration with time. In eCG treated and control females, the decline was significant ($p \leq 0.05$) in both 24 and 36 h. periods in comparison with 12 h period, but they showed no significant difference ($p \geq 0.05$) when compared with each other.

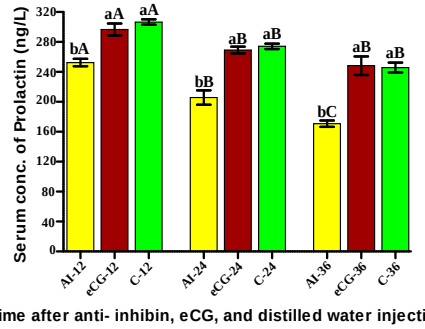

Figure (3): **Serum prolactin concentrations (ng/L) in AI and eCG treated female rats.**
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 IU 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.

Ir-Inhibin-B: figure (4) illustrates the results of serum ir-inhibin-B concentrations (ng/L) in virgin female rats after 12, 24, and 36 h of treatment. AI treated females revealed significant ($p \leq 0.05$) decline compared with eCG treated and control females at all periods, whereas eCG treated and control females recorded no significant ($p \geq 0.05$) differences after 12 and 24 h of treatment but they differ significantly ($p \leq 0.05$) after 36 h of treatment, where control level was higher than eCG treated level. In comparison between periods (12, 24, and 36 h) for each group, ir-inhibin-B concentration of AI group recorded significant ($p \leq 0.05$) gradual decrease with time progressing to be more significant ($p \leq 0.001$) at 36 h period. In contrast, eCG treated females showed no significant ($p \geq 0.05$) difference after 12, 24, and 36 h of treatment, whereas control females registered no significant ($p \geq 0.05$) difference between 12 and 24 h of treatment, but at 36 h period increased significantly ($p \leq 0.05$).

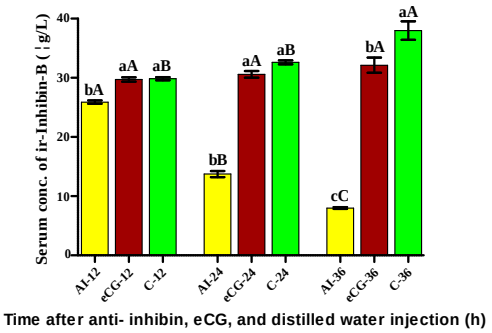

Figure (4): **Serum ir-inhibin B concentrations (ng/L) in AI and eCG treated female rats.**
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 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.

Estradiol-17B: the results of serum estradiol concentrations (ng/L) in virgin female rats, clarified in figure (5), revealed no significant ($p \geq 0.05$) differences among experimental groups after 12 h of treatment, while 24 h period recorded significant ($p \leq 0.05$) decline of serum estradiol concentration in eCG treated and control female rats compared with that recorder by AI treated females, where eCG treated and control groups showed no significant ($p \geq 0.05$) difference when compared with each other. After 36 h of treatment, serum estradiol concentrations in AI and eCG treated females showed no significant ($p \geq 0.05$) difference between each other, but they were significantly ($p \leq 0.05$) higher than control concentration. In comparison between periods (12, 24, and 36 h) for each groups, the concentration of AI treated group at 36 h was significantly ($p \leq 0.05$) higher than that of other periods (12 and 24 h), which showed no significantly ($p \geq 0.05$) difference when compared with each other. Estradiol levels of other female rats (control and eCG treated groups) recorded significant ($p \leq 0.05$) decrease at 24 h period compared with that of 12 h period, but they recorded further significant ($p \leq 0.05$) increase at 36 h period.

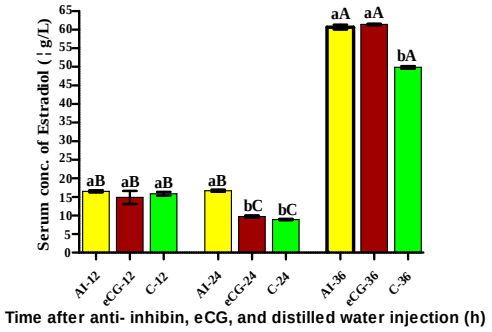

Figure (5): **Serum estradiol-17B concentrations (ng/L) in AI and eCG treated female rats.**
AI: female rats injected with 1 µg of AI/100 µl of dw/rat *ip*, at late metaestrus and 100 µl of dw *ip* after 54h. eCG: female rats injected with 20 IU of eCG/100 µl of dw /rat *ip*, in late metaestrus and 10 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.

Discussion

In virgin cycling female rats, AI treatment caused rapid significant decline of serum inhibin-B concentrations after 12 hours of treatment which continued in their decrease after 24, and 36 hours of treatment in comparison with that recorded by eCG treated and control females. This decrement could be attributed to immunoneutralization of endogenous inhibin caused by infusion of inhibin alpha antiserum. These results were in agreement with that reported previously by Abdulla (12) and

Thanoon (13). In contrast, this decrease accompanied by rapid increase of serum FSH and rapid decrease of prolactin concentrations (after 12 hours of treatment) and delayed increase of serum estradiol-17β concentrations (after 24 hours of treatment).

The decrement of serum inhibin-B concentration could allow activins to 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 (14).Scientific studies revealed that inhibin regulates FSH secretion by reducing the amount of activin available at the binding site and also by reducing activin binding with activin type II receptors (15). Activin binding to its receptors has been shown to increase FSH secretion (16), thus supposed our results where serum FSH concentration was significantly increased rapidly after 12 hours of AI treatment. 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 (14).

In comparison with eCG treated and control female rats, serum concentration of estradiol-17β in the AI treated female rats increased significantly after 24 hours and continued after 36 hours of treatment. This marked increment of estrogen levels occurred in concomitant with the notable growth of a large number of ovarian follicles in AI treated females after 24 hours and those treated with eCG after 36 hours 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 (5). These results clearly indicate that passive immunization against inhibin alpha subunit enhances biosynthesis of estrogens from ovarian follicles.

The improvement of immunoneutralization of female rat's endogenous inhibin, in the present study, was the significant elevation of serum FSH concentration along with that of serum estradiol-17β. 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. Increased FSH levels in AI treated female rats indicate that endogenous inhibin is a primary factor in the control of species-specific ovulation rate, mainly through the control of FSH secretion, as described previously in many species (17, 18).

In addition, treatment with eCG resulted in a dramatic increase in the secretion of inhibin and estradiol-17β. These increases were accompanied by a decrease in plasma levels of FSH as observed previously (19, 20). Our results indicate that stimulation of follicular development by administration of eCG was thought to induce an increase in plasma inhibin, which in turn suppress FSH secretion. Basal serum LH levels increased after 36 hours in AI and eCG treated females. This could be due to immunoneutralization of endogenous inhibin, as reported previously (6, 21, 22) and to eCG-hCG-treatment, which may be attributed to the cross reaction of exogenous eCG in the present LH receptors (LHRIA), which is in agreement with a previous study (23).

The present study reported decline of serum prolactin and elevation of estradiol-17β concentrations, as FSH secretion increased. The opposite correlation between estradiol-17β and prolactin concentrations may 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 (24).

It can be concluded that passive immunization against endogenous inhibin alpha subunit in virgin female rats, at late metastrus phase of estrus cycle, significantly increases the reproductive efficiency by modulate the reproductive hormonal profile compared with eCG-hCG treatment.

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التأثير المقارن لمضاد الانهبين وهرموني eCG-hCG على مستوى الهرمونات التكتثرية في إناث الجرذان الأباكير

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الخلاصة:
للتحقق من تأثير المعاملة بمضاد الانهبين وهرموني eCG-hCG على صورة الهرمونات التكاثرية في إناث الجرذان الأباكير، تم تم توزيع 54 إنثى جرذ أباكير منتظمة دورة الشبق بعمر 56 يوماً ومعدل ووزن تراوح بين 150- 170 غم عشوائيا على ثلاث مجموعات متساوية العدد (المجموعة المعاملة بمضاد الانهبين AI والمجموعة المعاملة بهرموني eCG-hCG والسيطرة C). تم حقن مجموعة AI بمضاد للانهبين (1 مايكروغرام لكل جرذ في الخلب) في نهاية طور ما بعد الشبق و حقنت 100 مايكروليتر من المحلول الفسلجي بعد 54ساعة، وحقنت مجموعة eCG بمحرض قند الفرس الحامل (20 وحدة دولية/ جرذ في الخلب) في نهاية طور ما بعد الشق وحقنت بمحرض قند البشر hCG (10 وحدات دولية/ حيوان في الخلب) بعد 54ساعة. وحقنت مجموعة السيطرة بالمحلول الفسلجي (100 مايكروليتر في الخلب) في نهاية طور ما بعد الشبق وبعد 54 ساعة. تمت التضحية بـ 13 إنثى (6 من كل مجموعة في كل 12ساعة ولثلاث مرات) وأخذت منها عينات دم لغرض قياس تراكيز الهرمون محفز الجريب FSH والهرمون المصفر LH والاستراديول والبرولاكتين والانهبين-ب). أظهرت نتائج الهرمون محفز الجريب تراكيز أعلى في مجموعة AI بالمقارنة مع المجاميع الأخرى خلال جميع مراحل التجربة ، كما كانت معدلات السيطرة أعلى معنويا من معدلات مجموعة eCG خلال تلك المراحل. سجلت تراكيز الهرمون المصفر تقارب معدلي مجموعتي AI والسيطرة إلا انهما كانا أعلى مما في مجموعة eCG فقط بعد 36 ساعة من المعاملة في حين تقارب معدلي ما بعد 12 و24 ساعة من المعاملة. خلال الحمل لم تظهر فروقات بين المجاميع عدا اليوم الأول الذي ظهر فيه معدل مجموعة eCG أعلى معنويا من المجموعتين الأخرتين. بينت نتائج تراكيز البروlاكتين انخفاضا معنويا في مجموعة AI من بين المجاميع الأخرى خلال مدد الدراسة قبل الحمل. خلال الحمل انعكست الصورة حيث كانت معدلات AI الأعلى تسجيلاً من بين المجموعات. إنخفضت تراكيز الانهبين معنويا في مجموعة AI بعد 12 و 24 و 36 ساعة من المعاملة بالمقارنة مع المجاميع الأخرى، بينما خلال الحمل ارتفعت التراكيز تدريجيا ووصلت الى درجة عدم المعنوية في اليوم الرابع من الحمل. سجلت تراكيز الاستراديول عدم وجود فروقات معنوية فيما بين المجاميع بعد 12 ساعة من المعاملة في حين ارتفع معدل مجموعة AI معنويا بعد 24 ساعة بالمقارنة مع المجموعتين الأخرتين. بعد مرور 36 ساعة كان معدلي مجموعتي AI و eCG متقاربين احصائيا، إلا انهما أعلى معنويا من السيطرة. خلال مدة الحمل، لم تظهر فروقات معنوية بين المجاميع ماعدا اليوم الأول الذي كان فيه معدل مجموعة AI أعلى معنويا من المجموعتين الأخرتين. من نتائج التجربة الحالية، يمكن الاستنتاج بأن التمنيع الميسر ضد الانهبين ألفا في أباكير إناث الجرذان يزيد من الكفاءة التكاثرية.
الكلمات المفتاح: مضاد الانهبين، eCG، الخصوبة، الهرمونات التكاثرية.