

Effect of steroid-free follicular fluid antiserum on reproductive endocrine profile at estrous and metestrus phases in female rats

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Abstract

Inhibins are important ovarian hormones that control the action of the pituitary gonadotropins which regulate the ovarian cycle. The present experiment aims to study the role of steroid-free follicular fluid-antibodies (S-FBFF-ab) on the reproductive hormone levels at estrus and metestrus phases in cycling female rats. Follicular fluid was collected from mature ovarian follicles, centrifuged and treatment with activated charcoal. S-FBFF was isolated and used for immunization of adult male rabbits. After 5 injections, blood was withdrawn for separation of S-FBFF-ab. Eighty mature female rats were assigned into control and treatment groups (40 each). At late metestrus phase, females of the control were injected intra-peritoneally with 100 µL of physiological saline, whereas treated females were injected intra-peritoneally with 100 µL of S-FBFF-ab. At early estrus and early metestrus phases of each estrous cycle, serum concentrations of inhibin-B, follicle stimulating hormone (FSH), estradiol (E2), and prolactin (PRL) were estimated. The results revealed that S-FBFF-ab has inhibin immuno-neutralization effect, which caused elevation in the concentrations of serum FSH and E2, and significant decline of serum PRL and inhibin-B, at early estrus, whereas significant elevation of E2 and PRL, and significant decline of serum FSH, at early metestrus. Furthermore, the results of gene expression revealed a significant elevation of ovarian aromatase gene at both early estrus and early metestrus phases, whereas pituitary PRL gene showed significant decline at early estrus phase and significant elevation at early metestrus phase. In conclusion, passive immunization against S-FBFF could augment the reproductive efficiency through increase reproductive endocrine activity.

Keywords: inhibin-B, follicular fluid, S-FBFF antiserum, FSH hypersecretion, immunoneutralization

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تأثير مضاد السائل الجريبي منزوع الستيرويدات على الصورة الصموية التكاثرية في أطوار دورة الشبق لإناث الجرذان

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الخلاصة

تعد الأنهيبيات من هرمونات المبيض المهمة التي تؤثر في فعل محرضات القند وتنظم الدورة المبيضية. أجريت الدراسة الحالية لدراسة تأثيرات مضاد السائل الجريبي منزوع الستيرويدات على صورة الهرمونات التكاثرية في طوري الشبق و metestrus لإناث الجرذان. تم سحب السائل الجريبي من الجريبات الناضجة وبعد تدويره بجهاز الطرد المركزي ومعالته بالفحم النشط، استخدم لتمنيع ذكور الأرانب. بعد خمس حقنات، تم سحب الدم وتدويره بجهاز الطرد المركزي للحصول على مضاد السائل الجريبي منزوع الستيرويدات. تم توزيع ٨٠ أنثى جرد باكر ومنتظمة دورات الشبق على مجموعتي حقنت بالبريتون في طور metestrus بالماء المقطر ١٠٠ مايكروليتر (السيطرة) و ١٠٠ مايكروليتر من مضاد السائل الجريبي منزوع الستيرويدات. في طوري الشبق و Metestrus، تم

تخدير ٢٠ أنثى من كل مجموعة وفي كل طور باستخدام الكيتامين والزايلازين ٠,٣ و ٠,١ ملليتر/كغم من وزن الجسم، على التوالي. سحبت نماذج دم من كل أنثى لقياس تركيز الأنهيبين والهرمون محفز الجريبب والأسترااديول والبرولاكتين. كما أخذت عينات من المبايض والنخامية لدراسة مستوى تعبير إنزيم الأروماتيز والبرولاكتين. أظهرت النتائج أن إناث المجموعة المعاملة حصل فيها تعادل مناعي للأنهيبين والذي رفع مستوى هرموني FSH والأسترااديول في المصل وانخفاض مستوي الأنهيبين والبرولاكتين في طور الشبق، بينما ارتفعت تراكيز الأسترااديول والبرولاكتين في طور Metestrus. كما أشارت نتائج الدراسة الجزيئية الى زيادة مستوى تعبير جين الأروماتيز للمبايض وفي كلا الطورين في حين انخفض تعبير جين البرولاكتين في النخام الغدي أثناء طور الشبق ثم ارتفع أثناء طور Metestrus. يستنتج أن التمنيع الميسر ضد السائل الجريبي منزوع الستيرويدات يزيد من خصوبة الإناث التكاثرية عن طريق زيادة إفراز الهرمونات التكاثرية.

Introduction

The endocrine systems play a main role in the estrous cycle. Various hormones involved are gonadotrophins (LH and FSH), estrogen and progesterone (1). FSH promotes folliculogenesis, while LH triggers ovulation in the ovary and develops corpus luteum (2). During the development of follicles, the level of estradiol is increased.

The rise in estradiol level also stimulates the part of adenohypophysis, therefore, the combination of effects on the hypothalamus and adenohypophysis will triggers the ovulatory surge of LH (3). Researchers have other choices to be induce superovulation, other than eCG-hCG treatment, in order to solve this problem in rats and immunizing against inhibin as in other animals. Multiple discoveries have proven that immunization against inhibins stimulates superovulation in both immature and adult rats (4-10).

It is conclusive that the inhibin antiserum treating method is advantageous for both oocyte and embryo production and quality (11-13). In vitro, using a follicular culture, many factors have been revealed to increase follicular growth and development, including antrum formation, where maybe they have direct or indirect stimulatory actions on follicular antrum development as a result of follicular growth (14). Inside the ovary, privately created local estrogens synthesis act together with the Gonadotropins emitted from the anterior pituitary gland to accommodate effective follicular growth and steroid hormones production (15). In the granulosa cells, it is a very much acknowledged that FSH is the real inducer of aromatase activity. Therefore, the stimulatory impact of FSH is liable to tweaks by various mixes. A rundown of a portion of these mixes and their activities on FSH initiated aromatase expression, where the role of estradiol, androgens, and insulin is the development of factor-I (15).

Our hypothesis is inducing reproductive hormones secretion by the supplementation of the S-FBFF-ab. Therefore, the aim of the current study was to determine the role of antisera against S-FBFF on reproductive hormones profile as well as ovarian aromatase and pituitary PRL gene expression levels in two consecutive estrous cycles of cycling female rats.

Materials and methods

Preparation of steroid-free bovine follicular fluid (SFBFF) antiserum

From bovine mature follicles (equal or more than 15 mm in diameter), the antral fluid was collected from slaughtered cow's ovaries, centrifuged at 8000 rpm for 15 minute, then the supernatant was treated for 90 min. with activated charcoal (10 mg/ ml), and after 60 minutes, centrifuged again at 14000 rpm for 90 min. at 4°C to discard the charcoal with attached steroids (12). The remaining S-FBFF was used to immunize 10 adult male rabbits by 5 injections of S-FBFF (100 µL/kg, one-week interval). One month after the fifth injection, blood was withdrawn, centrifuged to obtain S-FBFF-ab and kept at -20 °C (6).

Experimental animals

The present study was carried out in the agreement to the guidelines of ethics and policies of the University of Al-Qadisiyah. Adult cycling female Wistar rats (90 days old and 164.5± 5.17g weight) were housed under normal environmental conditions (12L: 12D daylight cycles and 22-24 °C temperature). The animals were fed on laboratory diet pellets (19% protein and 3000 kcal.) and drinking water *ad libitum*. Only female rats with estrous cycles of 4-5 days have been used. Estrus phases were determined according to the method described by Mandle *et al.* (16).

Experimental design

Eighty female rats were randomly assigned to treatment and control groups (40 each). The females of the control group were intra-peritoneally administered, at late metestrus stage, with a single dose of normal saline (100 µL), whereas those of treated group administered with S-FBFF-ab (100 µL). At early estrus and late metestrus phases, 20 females from each group at each phase were anesthetized with Ketamin and Xylazine (0.3 ml and 0.1 ml/ kg B.wt, respectively). Blood samples were withdrawn from the abdominal vein and blood sera were prepared. Serum inhibin-B, FSH, E2, and PRL concentrations were assessed using ELISA technique. The pituitary gland and ovarian tissue samples were obtained for assessment the

expression levels of prolactin gene and aromatase gene, respectively, using qRT-PCR technique (Bioneer, Korea). These genes were normalized by using housekeeping gene (GAPDH). This technique was done according to method described by Wang and Hardy (17).

Statistical Analysis

The results of experimental groups and periods were stated as mean \pm standard deviation ($M \pm SD$), and were compared by one-way analysis of variance (ANOVA1) and Newman-Keuls (18). In the current study, $P < 0.05$ was considered as a significant level in comparison the differences between the means. All statistical analysis was carried out using the GraphPad Prism- Version 5 (SAS Institute, Inc., USA).

Results

FSH

In comparison with control, serum FSH concentration of S-FBFF-ab treated females recorded significant ($P < 0.05$) increase at early estrus phase and significant ($P < 0.05$) decline at late metestrus phase. In comparison between the two phases, S-FBFF-ab treated females showed significant ($P < 0.05$) decline of serum FSH concentrations at metestrus phase in comparison with the early estrus phase, whereas control group female rats showed insignificant ($P > 0.05$) difference between the two phases (Table 1).

Estradiol

Serum estradiol concentration of S-FBFF-ab treated female rats revealed significant ($P < 0.05$) elevation in comparison with control females at both estrus and metestrus phases. In comparison between the two phases, S-FBFF-ab treated group females recorded significant ($P < 0.05$) elevation at metestrus phase compared with the estrus phase, whereas control group females showed no significant ($P > 0.05$) difference between the two phases (Table 1).

Prolactin

The result demonstrated in the table (1) showed significant ($P < 0.05$) decline of serum prolactin concentration in S-FBFF-ab treated females, at estrus phase, and significant ($P < 0.05$) elevation, at metestrus phase, compared with control females. Comparison between estrus and metestrus phases for each group, S-FBFF-ab treated group female rats recorded significant ($P < 0.05$) increase of prolactin concentration at metestrus in comparison with estrus phase, whereas the control group showed insignificant ($P > 0.05$) difference between the two phases.

Inhibin-B

Serum Inhibin-B concentration of S-FBFF-ab treated group female rats recorded significant ($P < 0.05$) decline compared with control females at the estrus phase, whereas second metestrus revealed insignificant ($P > 0.05$) difference between the means of the experimental groups. In comparison between the two phases, S-FBFF-ab treated group females showed significant ($P < 0.05$) decline at estrus phase compared with the metestrus phase, whereas control group females showed no significant ($P > 0.05$) difference between the two phases (Table 1).

Expression level of pituitary prolactin gene

Pituitary PRL gene expression level, illustrated in table 2, recorded significant ($P < 0.05$) decline in S-FBFF-ab treated females at estrus phase, and significant ($P < 0.05$) elevation at metestrus phase compared with control females. In comparison between the two phases for each group, S-FBFF-ab treated group female rats recorded significant ($P < 0.05$) increase of prolactin concentration at metestrus in comparison with estrus phase, whereas the control group showed insignificant ($P > 0.05$) changes between the phases.

Table 1: Serum reproductive hormone concentrations of control and S-FBFF ab treated female rats at early estrus and late metestrus phases of the estrous cycle

Hormones	At early estrus		At late metestrus	
	Control	Treatment	Control	Treatment
Inhibin B (ng/L)	51.65 \pm 6.330 Aa	6.430 \pm 1.011 Bb	48.72 \pm 5.001 Aa	46.52 \pm 5.684 Aa
FSH (IU/L)	9,383 \pm 1.096 Ba	39.88 \pm 2.918 Aa	8.615 \pm 0.983 Aa	5.015 \pm 0.677 Bb
E2 (ng/L)	18.31 \pm 2.981 Ba	32.56 \pm 3.817 Ab	17.91 \pm 2.346 Ba	49.71 \pm 5.012 Aa
PRL (ng/L)	210.9 \pm 11.11 Aa	145.3 \pm 10.92 Ab	220.8 \pm 14.56 Ba	315.5 \pm 23.87 Aa

C: control female rats injected with 100 μ l of normal saline *ip*, at late metestrus. T: treated female rats injected with 100 μ l/rat of steroid-free bovine follicular fluid antiserum *ip*, at late metestrus. Data were presented as Mean \pm SD of 15 observations (n=15). Different capital letters denote significant difference ($P < 0.05$) between groups for each phase. Different small letters denote significant difference ($P < 0.05$) between phases for each group.

Expression level of aromatase gene in the ovary

The ovarian aromatase gene expression level of S-FBFF-ab treated group was duplicated nearly 4 and 5 times than that recorded in control female ovaries at estrus and metestrus phases, respectively. In comparison between the

two phases, S-FBFF-ab treated females recorded significant ($P < 0.05$) elevation at metestrus phase compared with the estrus phase, whereas control females showed insignificant ($P > 0.05$) difference between the two phases (Table 2).

Table 2: Pituitary PRL and ovarian aromatase gene expression levels of control and S-FBFF ab treated female rats at early estrus and late metestrus phases of the estrous cycle

Gene	At early estrus		At late metestrus	
	Control	Treatment	Control	Treatment
Ovarian aromatase	8.764±0.516 Ba	23.40±1.31 Ab	9.236±1.03 Ba	42.45±3.23 Aa
Pituitary PRL	10.92±0.709 Aa	6.443±1.01 Bb	10.95±1.10 Ba	15.57±1.96 Aa

C: control female rats injected with 100 μ l of normal saline *ip*, at late metestrus. T: treated female rats injected with 100 μ l/rat of steroid-free bovine follicular fluid antiserum *ip*, at late metestrus. Data were presented as Mean \pm SD of 15 observations (n=15). Different capital letters denote significant difference ($P < 0.05$) between groups for each phase. Different small letters denote significant difference ($P < 0.05$) between phases for each group.

Discussion

The current study illustrated that S-FBFF-ab treatment caused a significant elevation in the levels of serum FSH and estradiol, at the early estrus phase, due to passive immunization against inhibin, by preventing inhibin from its negative feedback control on FSH secretion from the pituitary gland (9,19,20). This elevation could be the result of increased activin action on pituitary cells, producing gonadotropins, since stimulation the secretion of FSH is due to the functional antagonism of action between activin and inhibin, where inhibin counteracts the action of activin on the same receptors (11,21). Also, betaglycan could be another way, as it has a potent effect to increase the antagonistic action of inhibin against activin-mediated FSH release from the pituitary gland (22-24). Therefore, binding both types of inhibin's (A and B) with betaglycan is fundamental for their biological activity (25,26).

The results of several experiments reported that the females participated in the reproductive traits should be treated with the effectors, as the regulator of the secretion of the FSH, during the stage of early luteal phase (6,12,13), where the results in the current study showed high secretion of FSH as a result of immunoneutralization of endogenous inhibin. AL-Sa'aidi *et al.* (10) demonstrated the high level of estradiol after 12, 24 and 36 hours of immunization against endogenous inhibin in female rats. These results are consistent with the current study. Meanwhile, the immunoneutralization against endogenous inhibin, by infusion of S-FBFF-ab caused a rapid increase of pituitary FSH secretion and delayed increase of ovarian estradiol-17 β production. This finding agreed with the result of Al-Sa'aidi and Thanoon (20), where the decrement of serum inhibin concentration could allow activin to perform its action on pituitary gonadotrophs to secrete more FSH

because inhibins and activins are functionally antagonistic members (21).

The decreased serum reactive inhibin concentration due to passive immunization against endogenous inhibin, has been reported in the current study, which accompanied by increased serum levels of activin, FSH and estradiol have also reported by previous studies (27-30). In contrast, this decrease accompanied by a rapid elevation of serum FSH, rapid decrease of prolactin concentrations and delayed increase of serum estradiol-17 β concentrations. These findings were also reported by Al-Sa'aidi *et al.* (10) and Al-Shwielly (31). The decrement of reactive inhibin secretion could allow activins to perform its action on pituitary gonadotrophs to secrete more FSH (21). It has been mentioned that during pregnancy, serum prolactin concentration increased significantly in anti-inhibin treated females with the progression of pregnancy (32), and reach the highest value at the third trimester of gestation (33). They also reported activation of central prolactin receptor which suppresses LH surge through GABA/GABAAR signaling. These observations could explain the previous reports on the role of prolactin in modulating hypothalamic role in regulating mammalian reproduction (34).

A review of previous research on the relationship between growth factors, such as activins and inhibins, with prolactin secretion, we found that the relationship varied according to the functional status. Prolactin increases during pregnancy, which plays a synergistic role in the presence of growth factors to increase the proliferation of the glandular tissues of the mammary gland, especially in the growth of ducts and alveoli (32); whereas the role of prolactin is different during the estrus cycle, as it has been reported, in the current study, that passive immunization against inhibin, at late metestrus, decreased the secretion of prolactin from the anterior pituitary gland. These findings mention to the similar effect of inhibin and prolactin to

decrease the secretion of gonadotropin but in different ways, where inhibin acts directly on the gonadotrophs (7,22-24); whereas prolactin action is indirectly through hypothalamus by decreasing the secretion of hypothalamic gonadotropin releasing hormone (9,35).

Conclusion

It can be concluded that administration of S-FBFF-ab support reproductive efficiency by induction of reproductive hormones secretion, which can be applicable in the farm animals.

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Conflict of interest

We certify that there is no conflict of interest with any organization regarding the materials discussed in the manuscript.

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