Ovarian morphometric evolution in two consecutive estrous cycles of female rats treated with steroid-free bovine follicular fluid antiserum

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Abstract

The current study aims to investigate the role of steroid-free follicular fluid antiserum on ovaries morphometric growth and development in virgin female rats at two sequential estrous cycles. Bovine follicular fluid aspirated from graafian follicles, steroids were removed by treatment with activated charcoal, in order to obtained steroid-free bovine follicular fluid (S-FBFF) which was used for immunization of male rabbits to prepare S-FBFF antiserum (S-FBFF-ab). Sixty virgin female rats were assigned into control and treatment groups (30 each), intraperitoneal injected with a single dose of distilled water 100µl/rat and S-FBFF-ab 100µl/rat at late metestrus, respectively. At the estrus phase of the first and second estrous cycle, 15 females from each group of each cycle were anesthetized and ovarian samples were obtained for histological examination. In comparison with control, the results of S-FBFF-ab treated female rats revealed a significant increase of relative ovaries and uteri weights at both estrous cycles. Morphometric examination showed progressive ovarian proliferation at the first estrus phase in S-FBFF-ab treated female rats through elevation of the number of primaries, graafian, and total follicles. In conclusion, passive immunization against endogenous inhibin using S-FBFF-ab could augment the reproductive fecundity through increase ovarian growth and development.

Keywords: Inhibin, Passive immunization, Follicular fluid, Ovarian growth; Reproduction

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التطورات المبيضية الشكلية لدورتي شبق متتاليتين في إناث الجرذان المعاملة بمضاد السائل الجريبي منزوع الستيرويدات

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الخلاصة

أجريت الدراسة الحالية للتحري عن تأثير التمنيع ضد السائل الجريبي البقري منزوع الستيرويدات على التطورات الشكلية لمبايض إناث الجرذان في دورتي شبق متتاليتين. تم سحب السائل الجريبي البقري من جريبات كراف وتم التخلص من الستيرويدات عن طريق معاملة السائل مع الفحم النشط للحصول على السائل الجريبي البقري منزوع الستيرويدات الذي أستخدم في تمنيع ذكور الأرانب للحصول على مضاد السائل الجريبي البقري منزوع الستيرويدات. تم تقسيم 60 إناث جرذ باكر ومنتظمة دورات الشبق على مجموعتي سيطرة ومعاملة (30 لكل مجموعة). حققت الإناث المعالجة زيادة معنوية في الأوزان النسبية للمبايض والأرحام في كلتا الدورتين. وأظهر الفحص النسجي تطوراً ملحوظاً في نمو المبيضات في الدورة الأولى مع زيادة أعداد الجريبات الابتدائية وكراف.
Introduction

The commonly used method for induction superovulation in mice and rats is the treatment with combined equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG), but recently an alternative method was performed for this purpose (1-6). Many studies have used the alternative way in which the induction of superovulation can be attained and overcome the problems that occur in mice and rats. Furthermore, these studies have reported that an active or passive immunization against inhibin has the ability to induce superovulation in both the adult and immature mice and rats (1-11). Inhibin are glycoproteins and members of the transforming growth factor β (TGFβ) family. They are regarded as one of the major internal factors of the ovary that alter the role of the gonadotropins (Gn) as well as considered as one of the regulators of the ovary during the ovarian cycle (12,13). Moreover, inhibin is a potent regulator of FSH secretion by negative feedback mechanism through its endocrine impacts on adenohypophysis (14). Conversely, activins are decisive factors in the ovary, as they act through paracrine and autocrine and paracrine mechanism. Moreover, activins play direct roles in achievement of FSH secretion from the adenohypophysis (15). It has been found that FSH and the steroid in the follicular fluid modulate the expression levels of inhibin or activin subunit genes, their production and secretion (16). Administration of S-FBFF to intact heifers reduces the plasma FSH level and delays the ovulation onset, while termination of S-FBFF treatment re-established FSH secretion, where these effects are concluded to be mediated by inhibin was confirmed (17). New studies were conducted to improve the impacts of antisera against S-FBFF or inhibin-free S-FBFF on hormones profile in cycling female rats that affects reproduction (3,4).

The present study aims to investigate the impact of immunization against S-FBFF on ovarian growth and development, at two consecutive estrous cycles in virgin female rats.

Materials and methods

Preparation of steroid-free bovine follicular fluid antiserum (S-FBFFab)

Follicular fluid was collected from mature follicles ≤15 mm of bovine, centrifuged at 8000 rpm for 15 minutes to remove the debris, then after treated for 1 hour with activated charcoal (10 mg/ ml), and centrifuged again at 14000 rpm for 90 minutes to remove the charcoal with attached steroids. Proteins were detected using Biuret assay and ninhydrin reaction and cholesterol was estimated in the FF (18). Ten adult male rabbits were immunized against S-FBFF. One month after 5 injections (one-week interval), blood was collected, centrifuged and antiserum was obtained and kept at -20 °C (3).

Experimental animals

The current experiment was accorded by the ethical guidelines and policies of the University of Al-Qadisiyah. Adult virgin cycling female rats of Wistar strain (90 days old and 160-170 g of body weight) were housed at day light of 12L: 12D cycles and controlled temperature at 22-24 °C. Standard laboratory animal food (19% crude protein ratio and 3000 kilocalories energy) and drinking water was accessed ad libitum. Only female rats with regular estrous cycles have been used.

Experimental design

Sixty females were divided into two equal groups. The females were injected IP with a single dose of 100 μL of normal saline (control group) and 100 μL of S-FBFF antiserum (treatment group) at late metestrus phase. At the estrus phase of the first and second estrous cycle, 15 females from each group of each cycle were anesthetized by 0.3 ml Ketamine and 0.1 ml of Xylazine/ kg BW. Ovaries and uteri were removed and weighted. Ovarian samples were obtained for histological examination (19).

Statistical analysis

The results were presented as mean ± standard deviation (M±SD). Experimental groups and periods were compared using ANOVA-1 and Newman-Keuls (20). The significant level P<0.05 was considered as a significant to compare the differences between the means. All statistical analysis was carried out using the GraphPad Prism-Version 5 (SAS Institute, Inc., USA).

Results

As illustrated in figure 1, significant (P<0.05) elevation of relative ovarian weight in S-FBFF-ab injected female rats compared with control was registered at first cycle, whereas second cycle revealed insignificant (P>0.05) changes between the studied groups. In comparison between the two cycles for each group, S-FBFF-ab treated group female rats recorded significant (P<0.05) elevation of ovarian weight at first cycle in comparison with second cycle, whereas control group demonstrated no significant (P>0.05) changes between the cycles.
In comparison between periods for each group, S-FBFF-ab treated group recorded significant (P<0.05) elevation of uterine weight at the first cycle in comparison with second cycle, whereas control group showed no significant (P>0.05) difference between the two cycles.

Morphometric appearance (Figure 3T) and histological sections of ovaries from S-FBFF-ab treated females (Figure 4T), the results showed higher number of graafian follicles compared with that of control females (Figures 3C and 4C).

Histological sections from ovaries of treated group (Figure 4T) revealed positive role of S-FBFF-ab injection in ovarian tissue proliferation compared with control (Figure 4C), where it has been found an increment in tissue proliferation especially in number and size of follicles. Furthermore, the sections of treated female rats showed that the predominant type was the graafian follicles that contains oocytes and supporting tissue, as well as multilayered layer of proliferated granulosa cells and antrum filled with fluid (Figure 4T), whereas the sections of control females revealed lower number of graafian follicles and the predominant type of follicles were primary and secondary (Figure 4C).

The results presented in table 1, also confirmed these findings, where the number of primary and graafian follicles of S-FBFF-ab treated females increased significantly (P<0.05) compared with control.

In comparison between first and second estrus cycles for each group, the result of S-FBFF-ab treated group at first cycle (Figure 4T) recorded higher number of primary and graafian follicles and more proliferation than second cycle (Figure 5T), whereas control females showed insignificant differences between the two estrous cycles (Figure 4C and 5C) (Table 1).

In conclusion, the administration of S-FBFF-ab injection to cyclic female rats led to significant increase in uterine weight and number of graafian follicles compared with control group. This suggests a potential role of S-FBFF-ab in ovarian tissue proliferation and follicle development.

**Figure 1:** Relative ovary weight (g/100g of body weight) in cyclic female rats treated with steroid-free bovine follicular fluid at late metestrus phase. C: control female rats injected with 100 µl of normal saline ip, at late metestrus. T: treated female rats injected with 100µl/rat of steroid-free bovine follicular fluid antiserum ip, at late metestrus. Data were presented as Mean ±SD of 15 observations (n=15). Different capital letters denote significant difference (P<0.05) between groups for each cycle. Different small letters denote significant difference (P<0.05) between cycles for each group.

**Figure 2:** Relative uterus weight (g/100g of body weight) in cyclic female rats treated with steroid-free bovine follicular fluid at late metestrus phase. C: control female rats injected with 100 µl of normal saline IP, at late metestrus. T: treated female rats injected with 100 µl/rat of steroid-free bovine follicular fluid antiserum IP, at late metestrus. Data were presented as Mean ±SD of 15 observations (n=15). Different capital letters denote significant difference (P<0.05) between groups for each cycle. Different small letters denote significant difference (P<0.05) between cycles for each group.

**Figure 3:** Morphometric appearance of ovary obtained from SFBFF antiserum treated (T) female rats shows more developed and higher number of mature follicles in comparison with that of control (C).
Figure 4: Histological section of ovary obtained S-FBFF-ab treated (T1) female rat at first estrus cycle of the experiment shows obvious follicular growth and development and increased number of primary (PF) and graafian follicles (GF) with decreased number of secondary follicles (SF) in comparison with control (C1) female rats (H&E, x125).

Figure 5: Histological section of ovary obtained from S-FBFF-ab treated female rat at second estrus cycle of the experiment shows normal folliculogenesis and unchanged number of primary (PF), secondary (SF) and graafian follicles (GF) in comparison with control female rat (H&E, x125).

Table 1: Primary, secondary, mature and total ovarian follicles from control and SFBFF antiserum treated female rats

<table>
<thead>
<tr>
<th>Groups (Mean ±SD) n=15</th>
<th>C1st</th>
<th>T1st</th>
<th>C2nd</th>
<th>T2nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary follicles</td>
<td>10.2±0.78 b</td>
<td>13.5±0.86 a</td>
<td>9.98±0.97 b</td>
<td>10.1±1.02 b</td>
</tr>
<tr>
<td>Secondary follicles</td>
<td>8.65±0.68 a</td>
<td>6.88±0.84 b</td>
<td>8.26±0.75 a</td>
<td>8.44±0.83 a</td>
</tr>
<tr>
<td>Graafian follicles</td>
<td>8.54±0.83 b</td>
<td>12.7±0.88 a</td>
<td>9.13±0.92 b</td>
<td>9.85±0.75 b</td>
</tr>
<tr>
<td>Total follicles</td>
<td>27.3±2.76 b</td>
<td>33.8±2.16 a</td>
<td>27.4±3.06 b</td>
<td>28.3±3.11 b</td>
</tr>
</tbody>
</table>

C: control female rats injected with 100 µl of normal saline ip, at late metestrus. T: treated female rats injected with 100µl/rat of steroid-free bovine follicular fluid antiserum ip, at late metestrus. Different capital letters denote significant difference (P<0.05) between groups for each cycle. Different small letters denote significant difference (P<0.05) between cycles for each group.

Discussion

Various experiments confirmed the contribution of inhibin in female reproduction, as a regulator of FSH secretion from the adenohypophysis, is through the growth of dominant follicle during the luteal phase, which is normally associated with the high secretion of estradiol-17β (21). The overall activities of FSH in female reproductive system include ovarian granulosa cell proliferation and differentiation, gametes production, and secretion of inhibin and estrogens (22,23).
The significant increase in the relative weight of the ovaries and uteri of S-FBFF-ab treated group, at the first estrous cycle, could be attributed to the role of passive immunization against inhibin, which led to an increase in the rate of pituitary FSH secretion, which in turn stimulated the growth of ovarian follicles and the arrival of many of them into the stage of mature follicles (24). On the other hand, in parallel to increased folliculogenesis, estradiol secretion was also increased, since increased proliferation of granulosa cells has been also shown which is already responsible for estradiol secretion (25). It is known that estrogen plays a significant role in building fats and proteins as well as its role in the process of maturation of the ovary and its follicles and increase the level of certain types of proteins such as globulin binding proteins responsible for binding of sex hormones (26). One research demonstrated that progesterone and estrogen interaction increase rat uterine weight (27,28).

Furthermore, the increased level of growth hormone and insulin-like growth factors (3,29) could be the cause of increased ovarian weights, as insulin-like growth factors participates in the growth of follicles by stimulating the biosynthesis of DNA in granulosa cells and follicle cells (30,31). Pituitary activin (5,6) may also play an indirect role by influencing the secretion of FSH from the pituitary gland, as well as the direct effects of ovarian inhibin and activins on ovarian follicle growth and development (4,32,33).

The significant increase in the uter weight of S-FBFF antiserum treated group was accompanied by increased growth and development of ovarian follicles, which are responsible for the manufacture of a great amount of estrogens. On the other hand, estradiol is also known to have a stimulatory role in the maturation and development of the endometrium and increasing arterial blood flow (34). The fundamental role of estrogen is in the formation of endometrial tissue to be suitable for implantation of fertilized ova and the development of embryos until the development of placenta and embryonic membranes, which in turn secrete progesterone and estrogen (34). Furthermore insulin-like growth factors in the uterus are controlled by extracted estrogen from the implanted embryos, therefore it has been suggested that insulin-like factors may have a role in implantation (35) as well as to the role of activin to increase the number of estrogen receptors and improves its effectiveness (36).

Moreover, the histological sections from treated female rats revealed high proliferative rates of the ovaries. These coincide changes is due to the passive immunization against endogenous inhibin and consequent rise of FSH and estradiol secretion from the ovaries itself (37-39). In addition, the high level of growth hormone and insulin-like factors (4,41,42) may be the cause of increased ovarian tissue proliferation. Pituitary activin may also play an indirect role by influencing the discharge of FSH through the mechanism of the secretion through the adenohypophysis, as well as the direct effects of ovarian inhibin and activins that have localized effects on ovarian follicle formation (32,41,42).

The increased number of primary, graafian, and total ovarian follicles of treated females, due to the role of passive immunization against inhibin, which increases the concentration of FSH, where this hormone stimulates ovarian folliculogenesis and the production of a large amount of estrogens (25,39). Furthermore, growth hormone and IGFS have important roles in the granulosa cells proliferation, as growth hormone plays a major role in the release of growth promoters by increasing cell proliferation and inhibition of apoptosis (13,43).

Conclusion

It can be concluded that the prepared inhibin antiserum (from S-FBFF) was effective in immunoneutralization of inhibin, increasing the biosynthesis of pituitary FSH and ovarian estrogens production and increases ovarian growth and development, which could be efficient for improvement of female reproduction.

Acknowledgments

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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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