ISSN 1819-1878

Asian Journal of Animal Sciences

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OPEN ACCESS Asian Journal of Animal Sciences

ISSN 1819-1878
DOI: 10.3923/ajas.2017.183.188

Research Article
Effect of Immunization Against Steroid-Free Bovine Follicular Fluid on Reproductive Hormones Profile in Cycling Female Rats

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Abstract

Background and Objective: Inhibin is an intra-ovarian regulator of the ovarian cycle by modifying the action of the gonadotropins regulators of the ovarian cycle where Follicle Stimulating Hormone (FSH) secretion from anterior pituitary gland is dependent on the secretion of ovarian inhibin, estradiol or both of them. The present study was conducted, in cycling female rats to investigate the effect of antisera against steroid-free bovine follicular fluid (S-FBFF) or inhibin-free S-FBFF on hormones profile that influenced in reproduction. **Materials and Methods:** Follicular fluid was aspirated from bovine mature follicles, centrifuged and treated with activated charcoal. S-FBFF was divided into 2 parts: first part was used for immunization of adult male rabbits against S-FBFF (for obtaining inhibin antiserum; S-FBFF antiserum) and second part was treated with anti-inhibin before immunization of adult male rabbits against S-FBFF (for obtaining activin antiserum; IS-FBFF antiserum). After 5 injections (a week interval), blood was collected, centrifuged and antiserum was obtained. One hundred virgin cycling female rats (aged 65 days and weighted 150-170 g) were randomly assigned to five equal groups and i.p injected with 100 µL of normal saline (control), S-FBFF antiserum at proestrus (T1), IS-FBFF antiserum at proestrus (T2), S-FBFF antiserum at metestrus (T3), or IS-FBFF antiserum at metestrus (T4). At estrus phase, 10 females from each group were euthanized and blood samples were obtained for assessment of serum follicle stimulating hormone, estradiol, growth hormone and insulin-like growth factor-1 and -2 concentrations. One way analysis of variance (ANOVA) and Newman Keuls were used to test all groups unpaired values. **Results:** The results demonstrated significant increase of serum concentrations of FSH, estradiol-17β, GH, IGF-1 and IGF2 in T3 group among experimental groups, whereas the lowest ($p<0.05$) level was recorded by T4 group compared with control. **Conclusion:** In conclusion, inhibin immunoneutration can be inducted by the use of S-FBFF antiserum at metestrus, which has potent effect on reproductive potency in female rats.

Key words: Follicular fluid, inhibin, activin, follicle stimulating hormone, estradiol, insulin-like growth factors

Received: March 08, 2017 Accepted: May 27, 2017 Published: June 15, 2017

Citation: Alsaadi Jabbar Abbas Ahmed and Majhwol Ennas Mohammad, 2017. Effect of immunization against steroid-free bovine follicular fluid on reproductive hormones profile in cycling female rats. Asian J. Anim. Sci., 11: 183-188.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inhibins are members of the transforming growth factor β (TGFβ) family, which are structurally similar but functionally differ from other growth factors of the same family. Chemically, Inhibins are glycoproteins of two known types, inhibin-A and inhibin-B, where they composed of α-subunit linked to either βA-subunit or βB-subunit, respectively[1]. Inhibin is considered as one of the main intra-ovarian factors that modify the action of the gonadotropins and are involved in all steps of ovarian folliculogenesis and therefore, it considered as one of the intra-ovarian regulators of the ovarian cycle[2].

It has been postulated that Follicle Stimulating Hormone (FSH) secretion from anterior pituitary gland is dependent on the secretion of ovarian inhibin[3], estradiol[4] or both of them[5]. Kumanov *et al.*[6] mentioned that inhibin exerts its role through its autocrine, paracrine or endocrine effects on both gonadal and extra-gonadal tissues, where inhibin is a negative feedback regulator of FSH biosynthesis through its endocrine effects on glandular pituitary gland. On the other hand, activins have an important role in the ovary through their autocrine and paracrine action, as well as its direct role in increasing FSH secretion from the anterior pituitary[7]. Although, it has not been established that ovarian activins directly regulate pituitary FSH releasing, but it has been postulated that high levels of serum activin-A were found after inhibin immune-neutralization which accompanied by increased FSH secretion from the pituitary gland[8].

Inhibin was isolated from bovine follicular fluid[9] and it has been shown that FSH and the steroid follicular microenvironment differentially modulate the gene expression of inhibin/activin subunits, their assembly and secretion[10]. Many reports indicate that administration of steroid-free Bovine Follicular Fluid (BFF) to intact heifers decreases plasma FSH levels and delays the ovulation onset, whereas termination of bFF treatment is associated with a re-establishment of FSH hypersecretion. These effects were attributed to or mediated by inhibin[11].

The present study aimed to examine the effect of supplementation of prepared inhibin antiserum and activin antiserum, by injection of steroid-free bovine follicular fluid antiserum; SFBFF-Ab or inhibin- and steroid-free bovine follicular fluid antiserum; ISFBFF-Ab, respectively, at late metestrus on reproductive hormones levels in cyclic mature female rats. From this point a new knowledge can be attributed to the field of reproductive fecundity.

MATERIALS AND METHODS

The present study was conducted at the college of Veterinary Medicine, University of Al-Qadisiyah, during the period extended from October 15, 2016-February 25, 2016.

Collection and preparation of Follicular Fluid (FF): Follicular fluid was aspired from mature bovine ovarian follicles (≤15 mm in diameter). BFF was centrifuged at 8000 rpm for 15 min at 4°C to remove cellular debris. Activated charcoal (10 mg mL^{-1}) was added to the FF and mixed for 1 h at 4°C. Charcoal was removed by centrifugation at 14000 rpm for 90 min at 4°C. Charcoal treated FF was frozen at -20°C until use.

Detection of proteins in follicular fluid: Biuret assay and ninhydrin reaction has been used to detect the proteins according to Wise[12].

Estimation of cholesterol in charcoal treated FF: The cholesterol has been estimated in the Follicular Fluid (FF) according to Wise[12].

Preparation of steroid-free BFF and inhibin and steroid-free BFF antisera: S-FBFF was divided into 2 parts: first part was used for immunization of 10 adult male rabbits against S-FBFF (for obtaining inhibin antiserum; S-FBFF antiserum) and second part was treated with anti-inhibin before immunization of 10 adult male rabbits against S-FBFF (for obtaining activin antiserum; IS-FBFF antiserum). After 5 injections (a week interval), blood was collected, centrifuged at 14000 rpm for 30 min and antiserum was obtained and kept at -20°C until use.

Experimental animals: This study was approved for conducting laboratory rats in accordance to the ethical guidelines and policies of Al-Qadisiya University, Iraq. Virgin cycling female rats of Wistar strain (aged 65 days and weighted 150-170 g) were used in the present study. They were reared 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: One hundred cycling females were randomly assigned to five equal groups (control and four treatments). Control females were injected with normal saline (100 µL, ip) at proestrus. Treated groups were injected with 100 µL, ip of S-FBFF antiserum at proestrus (T1), IS-FBFF antiserum at proestrus (T2), S-FBFF antiserum at metestrus (T3) and IS-FBFF antiserum at metestrus (T4), respectively. At the fifth day, 10 females from each group were synthesized, by injection of 0.3 mL ketamine and 0.1 mL xylazine kg^{-1} b.wt.,) and blood samples were obtained for assessment of FSH, estradiol, IGF1, IGF2 and GH concentrations.

ELISA technique for hormonal assay in serum: Depending on the manufacturer instructions (ABO, Switzerland), serum hormones concentrations have been estimated.

Statistical analysis: All values were expressed as Mean±SD. Comparisons were performed using one way analysis of variance (ANOVA) and Newman-Keuls to test all groups' unpaired values[13]. Differences were considered to be significant at the level of $p<0.05$. All statistical analyses were carried out using the GraphPad Prism- Version 5 (SAS Institute, Inc., USA).

RESULTS

The results confirmed the potency of anti-inhibin injection (S-FBFF antiserum) in immunoneutralization of endogenous inhibin, particularly when used at metestrus phase, which accompanied by elevation of FSH, E2, GH, IGF-1 and IGF-2, whereas using the same antiserum at proestrus phase had no effect on the levels of these hormones. On the other hand, anti-activin injection (IS-FBFF antiserum) had a negative effect in both proestrus and metestrus phases.

As illustrated in Fig. 1, the results revealed that, in T3 group female rats, the concentration of serum FSH elevated ($p<0.05$) after injection of S-FBFF antiserum, at metestrus phase and declined ($p<0.05$) in T4 group female rats, after injection of IS-FBFF antiserum, at the same stage of estrus cycle, whereas injection of S-FBFF or IS-FBFF antiserum at proestrus phase, in T1 or T2 group female rats, respectively, had no effects ($p<0.05$) on the concentrations of serum FSH in comparison with control group female rats.

In T1 and T3 group female rats, the concentration of serum E2 increased ($p<0.05$) after injection of S-FBFF antiserum, at proestrus or metestrus phase, respectively and declined ($p<0.05$) in T4 group female rats, after injection of IS-FBFF antiserum, at metestrus phase of estrus cycle, whereas injection of IS-FBFF antiserum at proestrus phase, in T4 group female rats, had no effects ($p<0.05$) on the concentration of serum E2 in comparison with control group female rats (Fig. 1).

The concentration of serum GH in T3 group female rats elevated ($p<0.05$) after injection of S-FBFF antiserum at metestrus phase, whereas the concentrations in T1, T2 and T4 groups female rats, which immunized against S-FBFF prophase, IS-FBFF at prophase or IS-FBFF at metestrus, respectively, showed insignificant changes between each other and with that of control group female rats (Fig. 1).

After injection of S-FBFF antierum at metestrus phase, in T3 group female rats, the concentrations of serum IGF-1 and IGF-2 showed significant elevation ($p<0.05$), whereas injection of S-FBFF or IS-FBFF antiserum at proestrus, in T1 or T2 groups female rats, had no effects ($p<0.05$) on the concentrations of IGF-1 and IGF-2. In T4 group female rats, the injection of IS-FBFF deceased ($p<0.05$) the concentration of IGF-1 but had no effect ($p<0.05$) on the concentration of IGF-2 (Fig. 1).

DISCUSSION

The present findings revealed that immunoneutralization of inhibin using S-FBFF antiserum (T1) at proestrus phase has no effect on serum levels of hormones influenced by reproductive processes, whereas the immunization at late metaphase (T3) significantly $p<0.05$ increased the levels of FSH, estradiol-17β, GH, IGF-1 and IGF-2 in the blood. This result indicates that S-FBFF antiserum has inhibin immunonetralization impact, namely during late metaphase but not during prophase of the estrus cycle, where ovarian folliculogenesis, which is mainly dependent on FSH surge from pituitary gland, occurs at prophase[14]. On the other hand, in consequence to inhibin immunoneutralization, FSH level increased which could increases ovarian folliculogenesis[15]. Increased follicular growth and development, due to inhibin immunoneutralization and increased FSH level, could increase estradiol secretion from granulosa cells[16]. This finding was in agreement with that reported by Al-Shwilly[17].

In T2 and T4, the results reported decreased levels of the hormones. This finding indicates that IS-FBFF has no role to immunized endogenous inhibin, but instead the immunization might occur to the activin. This means that endogenous inhibin continued in the inhibitory effect on FSH secretion from the pituitary gland, which may be synergized by the immunization against activin, since activin and inhibin act in antagonized action on gonadotrophs in the pituitary gland[18].

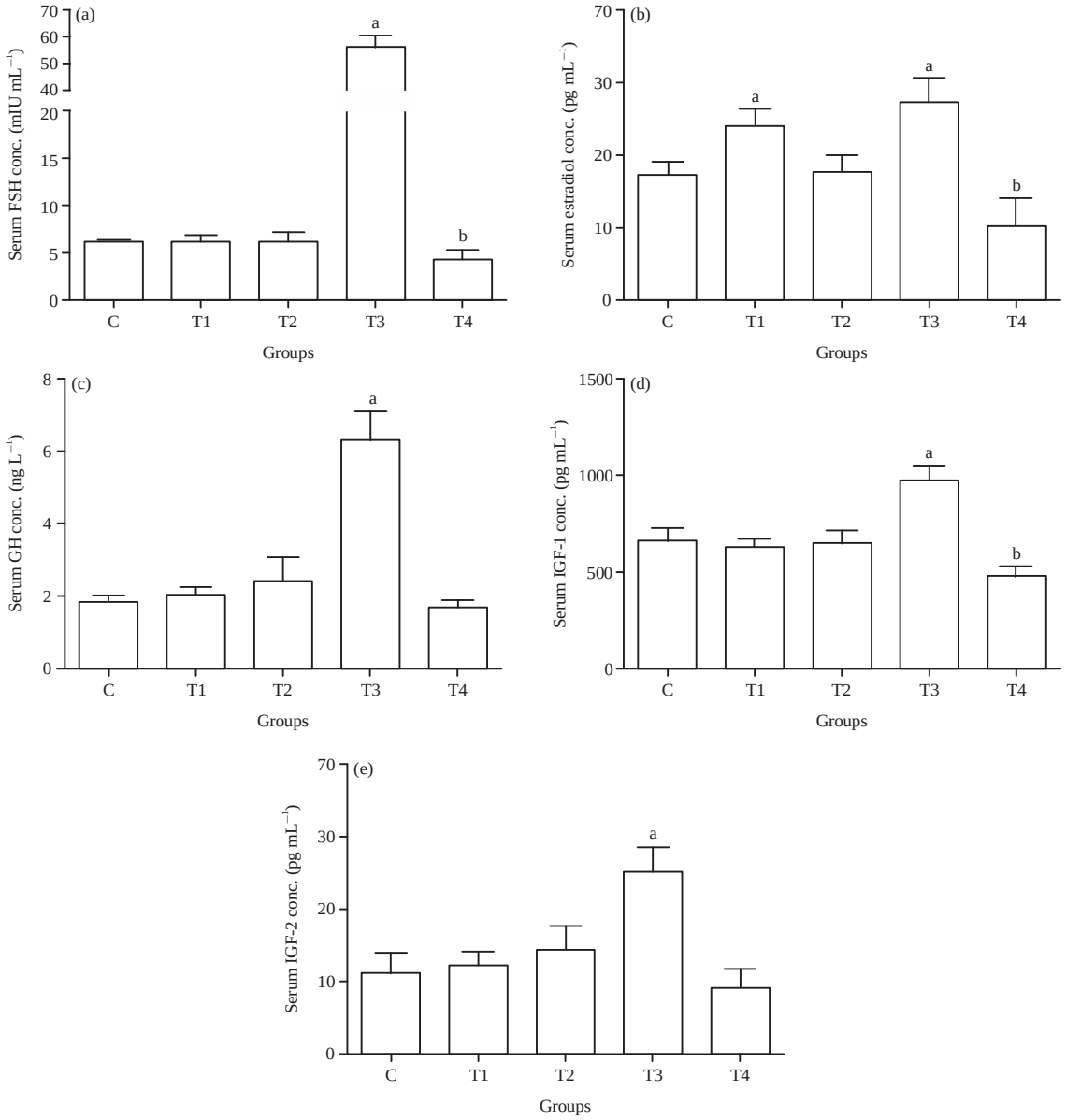

Fig. 1(a-e): Serum concentrations of (a) FSH (mIU mL^{-1}), (b) Estradiol-17β (pg mL^{-1}), (c) GH (ng L^{-1}), (d) IGF-1 (pg mL^{-1}) and (e) IGF-2 (μg L^{-1}) at estrus phase in cyclic female rats treated, at pro- or late metestrus phase, with SFBFF or ISFBFF antisera. Values presented as Mean±SD, the letters a and b denote significantly ($p<0.05$) higher and lower than control, respectively. C: 10 female rats, i.p injected with 100 μL of normal saline at proestrus phase, T1: 10 female rats, i.p injected with 100 μL of S-FBFF antiserum at proestrus phase, T2: 10 female rats, i.p injected with 100 μL of IS-FBFF at proestrus phase, T3: 10 female rats, i.p injected with 100 μL of S-FBFF antiserum at metestrus phase and T4: 10 female rats, i.p injected with 100 μL of IS-FBFF at metestrus phase

The increment of serum IGF-1 and IGF-2 levels in T3 group may be attributed to the high production these factors in gonadal and extragonadal tissues[19]. This finding is due to or as an indicator of increased GH secretion from the pituitary gland, where it has been postulated that GH act as a regulator of IGF-1 gene expression[20] as well as the role of increased estradiol level, which may also increases IGF-1 production from gonadal and/or extragonadal tissues. Al-Sa'aidi *et al.*[21] reported that passive immunization against inhibin alpha subunit accompanied by increased FSH and activin levels in female rats. Activin, on the other hand, causes increment of GH secretion from the pituitary gland[22]. It has been also

reported that IGF-1 and IGF-2 have an important role in steroidogenesis, either under endocrine effect or not, in the gonads[23], where it has been found that deletion of IGF-1 gene causes decreased steroidogenesis in the Lydig's cells of male rat testes[24]. The results of the present study could provide a new knowledge which can be implicated to assist reproduction of infertile women and also economically, it can be applicable in the improvement of farm animals fecundity.

CONCLUSION

It can be concluded that S-FBFF antiserum, when supplemented at metestrus, has immunoneutralization effect against endogenous inhibin, which causes elevation of serum FSH, estradiol, GH, IGF-1 and IGF-2 levels. This finding needs to be supported by other studies to examine the effect on ovarian functions. The present result might has an application approach in the future studies.

SIGNIFICANCE STATEMENT

The present study discovers the possible effect of prepared antisera against steroid free bovine follicular fluid as an anti-inhibin, on reproductive hormones that can be beneficial for improvement of reproductive performance in mammals. This study will help the researcher to uncover the area of passive immunization against endogenous inhibin of ovarian source. Thus passive immunization against follicular inhibin can be applicable for improvement of ovarian functions and reproductive fecundity.

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