Pituitary FSHβ and GH and Ovarian GH-r, IGF-1 and IGF-2 Gene Expression Levels in Cycling Female Rats Immunized against Steroid-Free Bovine Follicular Fluid Antisera

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ABSTRACT--- ***To examine the comparative effects of inhibin-free and/or steroid-free bovine follicular fluid antisera; IS-FBFF or S-FBFF, at proestrus or metestrus, on pituitary FSHβ and GH as well as ovarian GH-r, IGF-1 and IGF-2 gene expression levels, fifty cycling virgin female rats were randomly assigned equally to control and four treated group. Control females were intraperitonealy injected with 100 µl of normal saline at proestrus, whereas treated were intraperitonealy injected with 100 µl of S-FBFF at proestrus (T1), IS-FBFF at metestrus (T2), S-FBFF at metestrus (T3), and IS-FBFF at metestrus (T4). At the end of proestrus, females were euthanized; pituitaries and ovaries were dissected for assessment pituitary FSHβ and GH, as well as ovarian GH-r, IGF-1 and IGF-2 gene expression levels. The results illustrated significant increase of the expression levels of all studied genes in T3 group than others, whereas T4 group recorded the lowest levels among experimental groups. In T1 group, significant decrease has been shown for the expression levels of pituitary FSHβ and ovarian IGF-2 genes, and significant decrease of pituitary GH and ovarian IGF-1 genes, whereas T2 group recorded significant decrease of pituitary FSHβ and GH and ovarian IGF-2 genes and significant increase of IGF-1 gene expression in comparison with control. It can be concluded that immunization against S-FBFF could increase the expression levels that related to ovarian growth and proliferation.***

Keywords--- Pituitary, Follicular fluid, FSHβ gene, GH, IGFs, inhibin, activin.

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1. INTRODUCTION

Inhibin, as a member of the transforming growth factor β (TGFß) superfamily, is a main regulator of follicle stimulating hormone (FSH) secretion from gonadotrophs in the anterior pituitary gland, by a negative feedback mechanism, as well as its paracrine and autocrine effects on gonadal and extra-gonadal tissues[1] . Another member belongs to the same superfamily, activin acts in an antagonistic action to stimulate the secretion of pituitary FSH, either by its paracrine action at the pituitary level or by the action of activin of ovarian source in an endocrine mechanism[2].

In various studies, immunoneutralization of endogenous inhibin has been used to support gamete production and fertility[3-6]. It has been clearly demonstrated that immunization of different animals against inhibin has efficient role in increasing the ovulation rate, and induction of superovulation, which was associated with elevated plasma FSH concentrations, since immunoneutralization of endogenous inhibin act to diminish the negative feedback on the adenohypophyses leading to increased FSH secretion, enhanced follicular development and finally elevated ovulation rate in rats[3,7] , and uterine implantation sites and litter size in rats[8] .

Al-Obaidy *et al.*[9] reported that immunization of ewes against bovine follicular fluid (BFF) elevates ovulation rate without distinct increases in plasma concentrations of FSH, whereas Miller et al.[10] reported three-fold increase in FSH concentrations than control levels, with 40% increase in the ovulation rate.

In the current study we aimed to investigate the effect of prepared antiserum against circulating inhibin and activin together or against activin only, at proestrus or metaestrus, by injection of steroid-free BFF or inhibin and steroid- free BFF antisera, respectively, on pituitary FSHβ and GH and ovarian GH-r, IGF-1 and IGF-2 genes expression levels. As well as find out the critical time point of injection at proestrus or late metestrus.

2. MATERIALS AND METHODS

2.1. Collection and preparation of follicular fluid (FF):

Follicular fluid was collected by aspiration from mature bovine ovarian follicles (≤15mm in diameter). BFF was centrifuged, at 8000 rpm for 15 minute at 4˚C, treated with activated charcoal (10mg/ ml) and mixed for 1 hour at 4˚C. Charcoal was removed by centrifugation at 14000 rpm for 90 minute at 4˚C. Charcoal treated FF was frozen at -20˚C until use.

2.2. Preparation of antisera against steroid-free BFF and inhibin- and steroid- free BFF:

S-FBFF was divided into 2 parts: first part was used for immunization of 10 adult male rabbits against S-FBFF (for obtaining anti-inhibin or S-FBFF antiserum), and second part was treated with anti-inhibin before immunization of 10 adult male rabbits against S-FBFF (for obtaining anti-activin or IS-FBFF antiserum). One month after 5 injections (a week interval), blood was collected, centrifuged and antiserum was collected and stored at -20 ºC until use.

2.3. Experimental animals:

The approvement for conducting laboratory rats, in the present study, was under the accordance to the ethical guidelines and policies of AL-Qadisiya University, Iraq. Virgin cycling female rats of Wistar strain (aged 65 days and weighted 150-170g) were acclimatized to the controlled day light (12L: 12D cycles) and temperature (22-24 ºC) 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.

2.4. Experimental Design:

Fifty cycling females were randomly assigned to five equal groups (control and four treatments). Control females were supplemented with intraperitoneal injection of 100 µl normal saline at proestrus phase. Treated females were supplemented with intraperitoneal injection of 100 µl 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 end of proestrus, 10 females from each group were euthanized, pituitaries and ovaries were dissected and kept at -80 ºC until assessment of pituitary FSHβ and GH, as well as ovarian GH-r, IGF-1 and IGF-2 gene expression levels using qRT-PCR.

2.5. Quantitative Reverse Transcriptase Real-Time PCR:

According to the method mentioned in Wang and Hardy[11] , qRT-PCR technique was used for quantification of gene expression levels relative to Housekeeping genes in the pituitary gland and ovary. Data analysis of qRT-PCR assay included primer efficiency estimation and relative quantification of each gene expression level normalized by housekeeping gene expression (*GAPDH*). Threshold cycle numbers (Ct) were calculated from amplification plot of RT-PCR detection system, during exponential phase of fluorescent signals of SYBR®green primer the gene that react with cDNA of rat pituitary gland and ovary mRNA, where, the amount of DNA copy numbers (PCR product) in master mix reaction is doubles in each PCR cycle. First prepared series dilution of pituitary and ovary cDNA of control females were used with the primer of each gene to form the amplification plot, and from this amplification plot, threshold cycle (Ct) was used to calculate a linear regression based on the data points, and inferring the efficiency of each primer from the slope of the line. The relative quantification of target gene expression levels in pituitary gland and ovary have been calculated using the $2^{\wedge-\Delta\Delta Ct}$ livak and Schmittgen method. The expression of control gene was used as calibrator in both target and reference gene (*GAPDH*). At first, the threshold cycle number of target gene was normalized to that of reference gene in treatment groups and calibrator. Second, the ΔCt of treatment groups normalized to the ΔCt of calibrator, and finally the expression ratio (fold change) was calculated.

2.6. Statistical Analysis:

All values were expressed as mean ± SD. Comparisons were performed using one way analysis of variance (ANOVAI) and Newman- Keuls to test all groups' unpaired values. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis was carried out using the GraphPad Prism 5 (SAS Institute, Inc., USA).

3. RESULTS

The results showed significant ($p<0.05$) increase of pituitary FSHβ gene expression level in T3 group among experimental groups, whereas the lowest ($p<0.05$) level was recorded by T4 group compared with control, T1 and T2 groups. Pituitary GH gene expression levels of T3 and T1 were the highest ($p<0.05$) and of T2 and T4 were the lowest ($p<0.05$) in comparison with control. Ovarian IGF-1 gene expression level of T3 group was the highest ($p<0.05$) followed by T1 and T2 groups, whereas T4 group showed insignificant ($p>0.05$) difference compared with control. Ovarian IGF-2 gene expression level in T3 group was the highest ($p<0.05$) among experimental groups, whereas T1, T2 and T4 reported the lowest ($p<0.05$) levels compared with control. Ovarian GH-r gene expression levels of T3 and T1 were the highest ($p<0.05$) and of T2 and T4 were the lowest ($p<0.05$) in comparison with control (Figure 1).

4. DISCUSION

Findings of the present study clarify that immunization against steroid- free BFF at metestrus phase of cycling female rats has potent effects to elevates pituitary FSHβ and GH gene expression levels in the gonadotrophs and somatotrophes, respectively, which could be attributed to the absent of inhibitory effect of decreased inhibin and the stimulatory effect of high activin actions, as it has been postulated that the net of inhibitory or stimulatory effect was a result of the competition between activin and inhibin on the same ACT-I and ACT-II receptors[12-13]. These results were in agreement with that reported by Al-Sa'aidi and Al-Jayashi[14] , whom found an increase of FSHβ immunohistochemical expression levels in inhibin- and steroid-free BFF, and with that reported by Al-Sa'aidi and Thanoon[15] , whom reported increase of hypothalamic GHRH and pituitary GH gene expression levels in neonatal inibin immunoneutralized female rats.

Ovarian expression levels of GH-r, IGF-1 and IGF-2 increased significantly, in the present study, due to passive immunization against steroid-free BFF. These increments were in concomitant with the increment of pituitary FSHβ and GH gene expressions. These changes could be a result of decreased inhibin and increased FSH and activin concentrations. It has been mentioned that inhibins and activins perform various regulatory effects on gonads such as steroidogenesis, proliferation of granulosa cells and follicle growth, modulation of FSH receptors, and maturation of ova[16]. Ovarian Graffina's follicular fluid, which is a rich source of inhibins[17], has been tested to inhibit FSH secretion from anterior pituitary gland, as it has been found that passive immunization against bovine follicular fluid can induce superovulation by increasing FSH surge from anterior pituitary gland[18] . FSH is superintend for stimulating follicular growth and therefor rising estrogen output[19] . Then the higher concentrations of estradiol expressed with inhibin act to decline FSH secretion from the anterior pituitary gland by negative feedback reducing FSH to basal concentrations[20]. Estrogen inhibition effect is possibly through inhibition of GnRH production in negative feedback mechanism[21-22].

IGF-1 and IGF-2 are important in the function of almost every organ in the body[23]. From this point, the increase in ovarian IGF-I gene expression level could be attributed to the high activity of the ovaries of S-FBFF antiserum treated group, at metestrus phase, compared with other groups. On other hand, the convergence and decrease in the value of IGF-I in IS-FBFF could be related to the high level of inhibin and low level of FSH and activin and thereby estradiol concentration. A single injection of S-FBFF at metstrus phase in virgin Wistar female rats, also lead to significant increase of IGF-2 and GH-r gene expression levels compared with other groups of experiment. It has been reported that the increase in FSH concentration stimulate a large number of follicles to develop which lead to produce a large amount of estradiol[24]. The present findings were in agreement with that reported by Abdullah[25] who reported an increase of 9 and 16 times in IGF-1 and IGF-2 gene expression levels, respectively, in 45 days old female rats passively immunized against inhibin alpha subunit.

Previous studies postulated that immunization against endogenous inhibin results in high secretion of ovarian estradiol and activin, which also accompanying with high expression levels of gonadal GH-r[24,26]. In the present study it has been reported that ovarian expression of GH-r increased by infusion of S-FBFF antiserum. This increment may reflect in modulation of ovarian steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotropin secretion and responsiveness. The increment of ovarian GH-r expression, shown in the present study, may augmented by the high secretion of pituitary FSH, on the ovaries, since GH acts in conjunction with gonadotrophins to stimulate later stages of folliculogenesis[27]. The upregulation of *IGF*-I in ovarian tissues, found in the present study, may attributed to high secretion of GH from anterior pituitary, as it has been shown that GH rapidly activates *IGF-I* gene transcription and also regulates changes in chromatin structure within the *IGF-I* gene[28] . On the other hand, it has been demonstrated that activin A stimulated basal GH secretion via modulation of the transcription of the GH genes[29]. In addition to GH, other activators of *IGF* gene transcription include estradiol[30].

In conclusion, passive immunization of cycling female rats, at late metaphase, against S-FBFF increases the expression levels of pituitary FSHβ and GH genes as well as ovarian GH-r, IGF-1 and IGF-2 genes. From this point, the future research protocol can be directed toward field application in order to increase large animal's fecundity.

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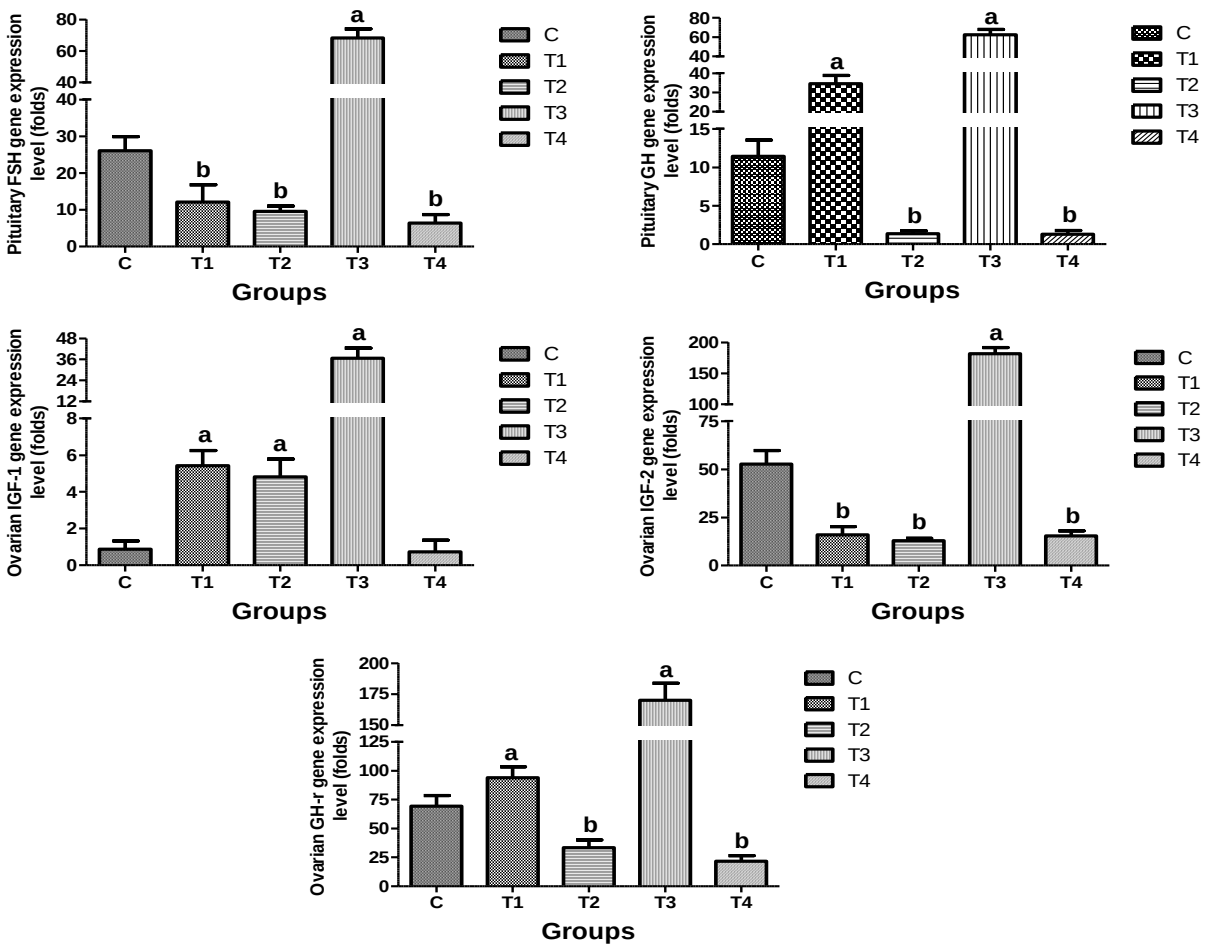

Figure (1): Expression levels of pituitary FSHβ and GH and ovarian GH-r, IGF-1 and IGF-2 genes at estrus phase in cycling female rats treated, at proestrus or metestrus phase, with S-FBFF or IS-FBFF.

Values presented as M±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.

T4: 10 female rats, i.p injected with 100 µl of IS-FBFF at metestrus phase.