Adenohypophyseal Immunohistochemoial Expression Levels of FSHβ in Cyclic Virgin Female Rats Treated with Steroid Free Bovine Follicular Fluid Antiserum

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Abstract

The present study was conducted to investigate the role of antisera against steroid-free bovine follicular fluid (SFBFF) or inhibin-free SFBFF on he pituitary immunohistochemical expression levels of FSHβ in cyclic virgin female rats. Follicular fluid was aspirated from bovine Graffian follicles (>15 mm in diameter), centrifuged to remove cellular debris, treated with activated charcoal. SFBFF was divided into 2 parts: first part was used for immunization of rabbits against SFBFF (for obtaining anti-inhibin SFBFF; AI-SFBFF), and second part was treated with anti-inhibin before immunization of rabbits against SFBFF (for obtaining anti-activin SFBFF; AA-SFBFF). After 5 injections (a week interval), blood was collected, centrifuged and antiserum was obtained. After estrus synchronization, 120 adult virgin female Wistar rats (aged 56 days and weighted 146±4.35 g) were randomly assigned into three equal groups (40 females each) and injected intrperitonealy, at early proestrous, with 100 µl of normal saline (control), 100 µl of AI-SFBFF (AI-SFBFF group), and 100 µl of AA-SFBFF (AA-SFBFF group). Estrus cycle phases have been monitored. At each phase of the cycle, 10 females from each group were cascificed, dissected and the pituitaries were obtained for immunohistochemical expression density of FSHβ. The results demonstrated positive immunostaining of FSHβ at all stages of estrus cycle with differences between groups, where AI-SFBFF group showed higher expression levels of FSHβ, at both proestrus and estrus phases, among experimental groups. In conclusion, the ovarian source of inhibin has negative feedback actions on pituitary FSH, whereas ovarian source of activin has FSH releasing endocrine role as well as its autocrine and paracrine effects as growth promoter.
KEYWORDS: Pituitary, Follicular fluid, Inhibin, Activin, FSH.

INTRODUCTION

Inhibin, as a member of the transforming growth factor β (TGFß) superfamily, is a main regulator of follicle stimulating hormone (FSH) from the adenohypophesis, as well as its paracrine and autocrine effects on gonadal and extragonadal tissues (Kumanov *et al.,*2005). In variou studies, immunoneutralization of endogenous inhibin has been used to support gamete production and fertility (Rivier and Vale, 1989; Ishigame et al., 2004; 2005; Al-Sa’aidi and Samir, 2010; Samir, 2010; Al-Sa’aidi et al., 2013).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 mice (Medan et al., 2004), rats (Rivier and Vale, 1989; Al-Sa'aidi and Samir, 2010; Samir, 2010), guinea pigs (Shi et al., 2000), hamsters (Kishi et al., 1997), ewes (Maan et al., 1993; Russell *et al.,* 1994; Tannetta *et al.,* 1997), goats (Medan et al., 2004), cows (Takedomi et al., 1997), and mares (Nambo et al., 1998),and uterine implantation sites and litter size in rats (Al-Sa'aidi et al., 2016).Al-Obaidy *et al.* (1987) 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. (1982) 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 role of prepared antiserum against circulating inhibin and activin together or against activin only by injection of steroid-free BFF or steroid and inhibin- free BFF, respectively, and examine their effects on pituitary immunohistochemical expression levels of FSHβin cycling female rat.

MATERIALS AND METHODS

Collection and preparation of follicular fluid (FF)

Follicular fluid has been aspired from bovine ovarian follicles (≤15mm in diameter). BFF were centrifuged at 8000 rpm for 15 minute at 4˚C to remove cellular debris. Activated charcoal (10mg/ ml) was added to the FF 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. It is reported that 99% of the original steroids were removed by this technique.

Detection of proteins in follicular fluid: Biuret assay and ninhydrin reaction has been used to detect the proteins in the FF (Wise, 1987).

Estimation of cholesterol in charcoal treated FF the cholesterol has been is estimated in the FF according to Wise (1987).

Preparation of sterid-free BFFand steroid- and inhibin-free BFF antisera: SFBFF has been divided into two parts, first part was used for immunization of rabbits against SFBFF (for obtaining anti-inhibin SFBFF; AI-SFBFF), and second part was treated firstly with anti-inhibin and used for immunization against SFBFF (for obtaining anti-activin SFBFF; AA-SFBFF). For each part, five mature male rabbits have been injected with 1 ml. of SFBFF or with 1 ml. of anti-inhibin treated SFBFF (sc.) for 5 times (one week interval). One month after the last injection, blood was collected, centrifuged and antiserum was obtained and stored 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 female Wistar rats (aged 56 days and weighted 146±4.35 g) have been 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:after estrus synchronization, daily vaginal smears for all female rats were performed for 4-5 consecutive days to detect the phases of estrus cycle. One hundred and twenty female rats were randomly assigned to three equal groups (control

and two treatment groups).At early proestrous, female rats were intraperitonealy injected with100 µl of normal saline (Control group), 100 µl of AI-SFBFF (AI-SFBFF group), and 100 µl of AA-SFBFF (AA-SFBFF group).Estrus cycle phases have been monitored. At each phase of the cycle, 10 females from each group were anesthetized (by injection of 0.4 ml of thiopentalsod./ animal), dissected and the pituitarieswere removed and fixed in formalin 10% for immunohistochemical examination to determine the expression density of FSHβ.

Histological study: histological sections have been orepared according to Luna (1968).

Immunohistochemistry-Paraffin protocol: according to the manufacture instructions (**www.abcam.com/technical**), immunohistochemistry (IHC) was performed.

Statistical Analysis: all values were expressed as mean ± SEM. Comparisons were performed using two way analysis of variance (ANOVAII) 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 were carried out using the GraphPad Prism (SAS Institute, Inc., USA).

RESULTS

The result clarified in figure (2) reveals the effect of treatment with steroid free bovine follicular fluid antiserum (anti-inhibin SFBFF) or inhibin and steroid free bovine follicular fluid antiserum (anti-activin SFBFF) on qualitative scoring of immunohistochemical expression of FSH in the pituitary tissues at different stages of the estrus cycle in cyclic virgin female rats. At proestrus (figure 1-A and table 1) and estrus (figure 1-B and table 2) phases of the estrus cycle, the result reveals that AI-SFBFF treated female pituitaries registered higher scores ($p<0.05$) of positive cells and intensity of staining, whereas AA-SFBFF female pituitaries registered lower score ($p<0.05$) in comparison with control females. While metestrus (figure 1-C and table 3) and diestrus (figure 1-D and table 4) phases registered no significant differences ($p>0.05$) between experimental groups. In comparison between phases of the estrus cycle, all experimental groups recorded higher FSH immunohistochemical expression denity ($p<0.05$) at proestrus and estrus phases, whereas the lower expression densities have been reported at metestrus and diestrus phases (figure 2).

DISCUSION

Transforming Growth Factor-β (TGF-β) signaling controls various cellular processes, such as cell proliferation, recognition, differentiation, and apoptosis, during embryogenesis as well as in mature tissues of mammals. (Massague et al., 2000). Inhibins and activins are members of TGF- β superfamily with antagonistic actions. They regulate various sets of functions, involving reproductive performance and efficiency (deJong, 1988).

In the present study, the prepared inhibin and activin antibodies from bovine follicular fluid, as it has been mentioned that ovarian Graffian's follicles are good source for these peptides (Nashimoto et al., 2009), have been tested to clarify the role of ovarian activin on FSH secretion from anterior pituitary. To answer this question, two types of antibodies have been prepared in the current study. The first type of antibodies has been prepared by injecting adult male rabbits (five times with one week interval) with steroid-free bovine follicular fluid, to get antisera against endogenous inhibin (AI-SFBFF group) and to let activin to perform its stimulatory effect on FSH secretion from the anterior pituitary without antagonistic effect of inhibin, whereas the second type of antibodies has been

prepared by injecting the male rabbits with both inhibin- and steroid-free bovine follicular fluid, to get antisera against endogenous activin (AA-SFBFF group) and to let endogenous inhibin performs its inhibitory action on FSH secretion from the anterior pituitary without antagonistic effect of activin.

Therefore, the current study could be useful in the investigation of the in vivo roles of these factors in ovaries, as autocrine and paracrine actions, and pituitaries, as endocrine action, by investigationg the immunohistochemical staining density of pituitary FSH density. As it has been well evidenced that dysfunction of inhibin and/or inhibin-activin antagonism disorder may result in infertility (Shelling *et al.*, 2000; Marozzi *et al.*, 2002). Therefore, illuminating the full picture of ovarian inhibin and activin effects will possibly provide important knowledge about the activity of ovarian function. Similarly, the possible regulatory role of inhibins and activins in ovarian cellular growth and development and steroidogenesis could provide new tools to increase fertility and reproductive outcome in the future.

In AI-SFBFF treated females, the high density of pituitary FSH could be attributed to the high secretion of FSH from gonadotrophes due to the immunoneutralization of endogenous inhibin by the prepared inhibin antibodies. These events could be due to the inhibitory action of inhibin and/or the stimulatory action of activin, where it has been postulated that the net inhibitory or stimulatory effect was a result of the competetion between inhibin and activin on the same gonadotrophes type II and type I receptor interaction sites (Nickel *et al*., 2001; Hart *et al*., 2002; Thompson *et al*., 2003; Harrison *et al*., 2004). Activin stimulates the secretion of FSH through its binding with high affinity to ActRII or ActRIIB receptors that found at the surface of adenohypophesial gonadotrophs (Shi and Massague, 2003). Inhibin can binds both ActRII and ActRIIB receptors with a higher affinity than activin (Lewis *et al*., 2000).

In AA-SFBFF group, the prepared anti-activin could immunoneutralize the local activins produced inside the pituitaries. This immunoneutralization against activin will reliefs activin action and allows inhibin to performe its inhibitory action on pituitary gonadotrophes. Therefore there is very low IHC density of FSH in the pituitaries of AA-SFBFF treated female rats. From these findings, it can be postulated that antibodies against ovarian activin has an immunoneutralization effects against pituitary activins, as inhibin performed its inhibitory action on gonadotrophes without antagonistic effect of pituitary activin. Meaningwhile activin stimulatory effect has been removed by AA-SFBFF treatment.

Previos studies demonstrated that passive immunization of female rats against inhibin-α subunit increases the concentration of plasma FSH (Culler and Negro-Vilar, 1988; Thanoon, 2013; Abdulla, 2013) and elevates the ovulation rate (Rivier and Vale, 1989; Al-Sa'aidi and Samir, 2010). Also, plasma levels of estradiol-17β significantly increased in the female rats treated with inhibin-antiserum (Thanoon, 2013). It has been suggested that a high level of endogenous FSH stimulates the wave of follicular development and results in production of a large amount of estradiol-17 β, which induces the LH surge by positive feedback effect to the hypothalamus-pituitary axis (Taya, 1993; Taya and Watanabe, 1999).

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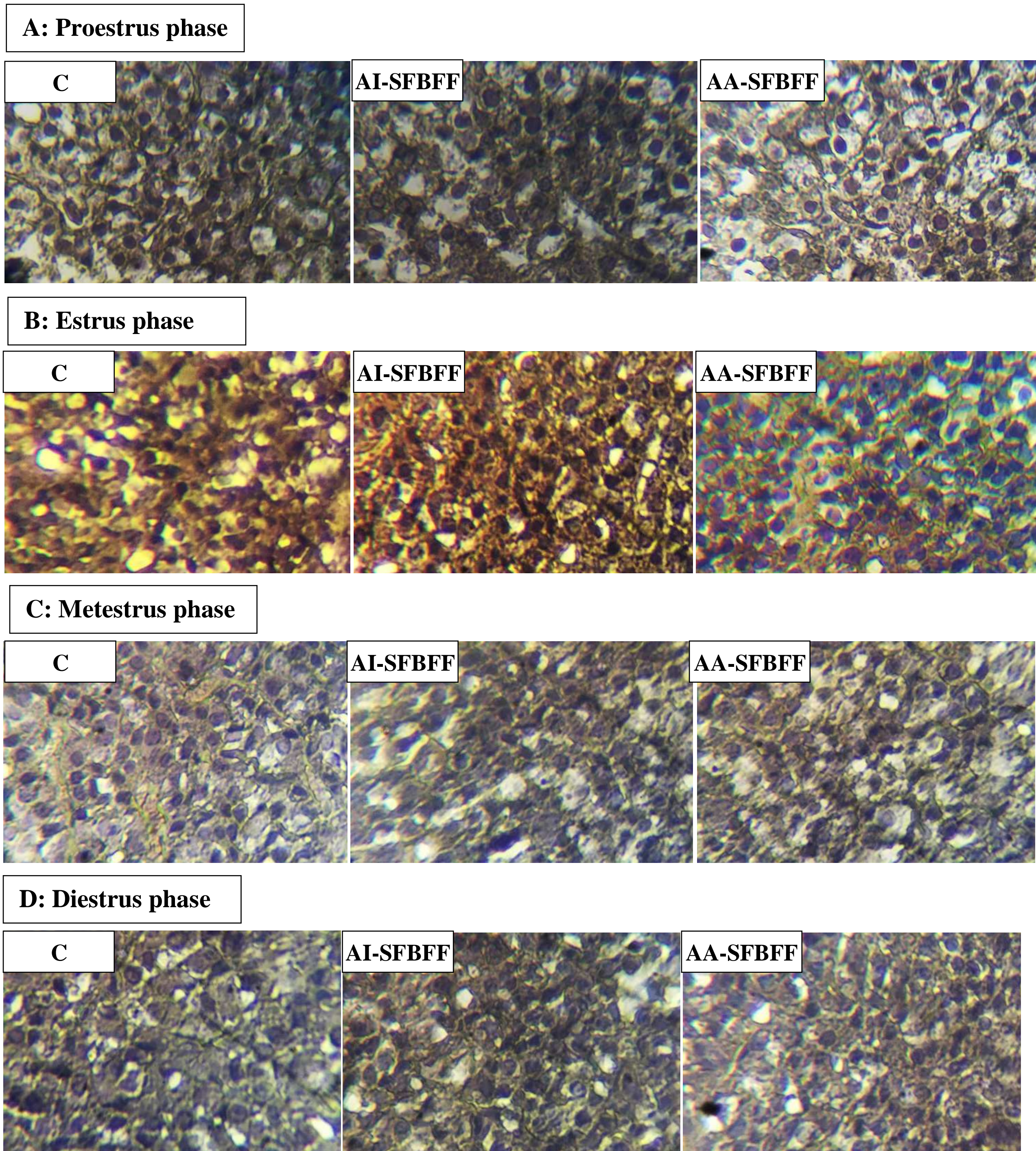

Figure (1): histological sections obtained from adenohypophysis show FSH immunohistochemical densities in experimental groups. AI-SFBFF group reveales higher density, whereas AA-SFBFF group revealed lower density of FSH inside gonadotrophes (Gn) and interstitium (IS) compared with control, at proestrus (A), estrus (B), and no marked differences in immunohistochemical density of FSH between experimental groups at metestrus (C) and diestrus (D). IHC stain 400x.

Table (1): Qualitative scoring of FSH IHC in adenohypophysis at proestrus phase.

<table>
<tr><td colspan="2">Score</td><td colspan="3">0</td><td colspan="3">1+</td><td colspan="3">2+</td><td colspan="3">3+</td><td rowspan="4">Q = P*I</td></tr>
<tr><td colspan="2">Positive Cells (P)</td><td colspan="3"><10%</td><td colspan="3">10-25%</td><td colspan="3">25-50%</td><td colspan="3">50-75%</td></tr>
<tr><td colspan="2">Score</td><td colspan="4">1</td><td colspan="4">2</td><td colspan="4">3</td></tr>
<tr><td colspan="2">Intensity of Staining (I)</td><td colspan="4">weak staining</td><td colspan="4">Moderate staining</td><td colspan="4">strong staining</td></tr>
<tr><td rowspan="2">C-1</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">55</td><td rowspan="2">55*2=110</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">C-2</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">58</td><td rowspan="2">58*2=116</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">C-3</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">55</td><td rowspan="2">55*2=110</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">C-4</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">51</td><td rowspan="2">51*2=102</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">C-5</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">32</td><td colspan="3"></td><td rowspan="2">32*2=64</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td colspan="14">Mean ± S.E.</td><td>100.4±7.1 1 b</td></tr>
<tr><td rowspan="2">AI-SFBFF-1</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">42</td><td colspan="3"></td><td rowspan="2">42*3=126</td></tr>
<tr><td>1</td><td colspan="4"></td><td colspan="4"></td><td colspan="4">3</td></tr>
<tr><td rowspan="2">AI-SFBFF-2</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">65</td><td rowspan="2">65*2=130</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">AI-SFBFF-3</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">70</td><td rowspan="2">70*3=210</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4"></td><td colspan="4">3</td></tr>
<tr><td rowspan="2">AI-SFBFF-4</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">80</td><td rowspan="2">80*3=240</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4"></td><td colspan="4">3</td></tr>
<tr><td rowspan="2">AI-SFBFF-5</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3"></td><td colspan="3">52</td><td rowspan="2">52*3=156</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4"></td><td colspan="4">3</td></tr>
<tr><td colspan="14">Mean ± S.E.</td><td>172.4±12. 7a</td></tr>
<tr><td rowspan="2">AA-SFBFF-1</td><td>P</td><td colspan="3"></td><td colspan="3">15</td><td colspan="3"></td><td colspan="3"></td><td rowspan="2">15*2=30</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">AA-SFBFF-2</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">38</td><td colspan="3"></td><td rowspan="2">38*2=76</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
<tr><td rowspan="2">AA-SFBFF-3</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">38</td><td colspan="3"></td><td rowspan="2">38*1=38</td></tr>
<tr><td>I</td><td colspan="4">1</td><td colspan="4"></td><td colspan="4"></td></tr>
<tr><td rowspan="2">AA-SFBFF-4</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">30</td><td colspan="3"></td><td rowspan="2">30*1=30</td></tr>
<tr><td>I</td><td colspan="4">1</td><td colspan="4"></td><td colspan="4"></td></tr>
<tr><td rowspan="2">AA-SFBFF-5</td><td>P</td><td colspan="3"></td><td colspan="3"></td><td colspan="3">35</td><td colspan="3"></td><td rowspan="2">35*2=70</td></tr>
<tr><td>I</td><td colspan="4"></td><td colspan="4">2</td><td colspan="4"></td></tr>
</table>

Mean ± S.E.	48.8±5.3 c

Different letters represent significant difference compared with control (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
AI-SFBFF= virgin female rats injected with 100 µl of anti-inhibin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.
AA-SFBFF= virgin female rats injected with 100 µl of anti-activin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.

Table (2): Qualitative scoring of FSH IHC in adenohypophysis at estrus phase.

Score		0	1+	2+	3+				Q = P*I
Positive Cells (P)		<10%	10-25%	25-50%	50-75%				
Score						1	2	3	
Intensity of Staining (I)						weak staining	Moderate staining	strong staining	
C-1	P				50				50*2=100
	I						2		
C-2	P			45					45*2=90
	I						2		
C-3	P				60				60*2=120
	I						2		
C-4	P				51				51*2=102
	I						2		
C-5	P				55				55*2=110
	I						2		
Mean ± S.E.									104.4±3.8 b
AI-SFBFF-1	P				50				50*3=150
	1							3	
AI-SFBFF-2	P				65				65*2=130
	I						2		
AI-SFBFF-3	P				75				75*3=225
	I							3	
AI-SFBFF-4	P				80				80*3=240
	I							3	
AI-SFBFF-5	P				55				55*3=115
	I							3	
Mean ± S.E.									172.0±13.5a
AA-SFBFF-1	P		20						20*2=40
	I						2		

AA-SFBFF-2	**P**			**40**		**40*2=80**
	I		**2**			
AA-SFBFF-3	**P**			**40**		**40*1=40**
	I	**1**				
AA-SFBFF-4	**P**			**30**		**30*2=60**
	I		**2**			
AA-SFBFF-5	**P**		**22**			**22*2=44**
	I		**2**			
Mean ± S.E.						**52.8±6.4 c**

Different letters represent significant difference compared with control (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
AI-SFBFF= virgin female rats injected with 100 µl of anti-inhibin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.
AA-SFBFF= virgin female rats injected with 100 µl of anti-activin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.

Table (3): Qualitative scoring of FSH IHC in adenohypophysis at metestrus phase.

Score		**0**	**1+**	**2+**	**3+**	**Q = P*I**
Positive Cells (P)		**<10%**	**10-25%**	**25-50%**	**50-75%**	
Score		**1**	**2**	**3**		
Intensity of Staining (I)		**weak staining**	**Moderate staining**	**strong staining**		
C-1	**P**		**20**			**20*2=40**
	I		**2**			
C-2	**P**		**22**			**22*2=44**
	I		**2**			
C-3	**P**		**24**			**24*2=48**
	I		**2**			
C-4	**P**		**18**			**18*2=36**
	I		**2**			
C-5	**P**			**27**		**27*2=54**
	I		**2**			
Mean ± S.E.						**44.4±2.8a**
AI-SFBFF-1	**P**			**42**		**42*2=84**
	1		**2**			
AI-SFBFF-2	**P**		**24**			**24*2=48**
	I		**2**			
AI-SFBFF-3	**P**			**45**		**45*1=45**
	I	**1**				
AI-SFBFF-4	**P**		**23**			**23*2=46**

	I		2			
AI-SFBFF-5	P		20			20*2=40
	I		2			
Mean ± S.E.						52.6±5.6 a
AA-SFBFF-1	P		15			15*2=30
	I		2			
AA-SFBFF-2	P		22			22*2=44
	I		2			
AA-SFBFF-3	P		20			20*2=40
	I		2			
AA-SFBFF-4	P		30			30*1=30
	I	1				
AA-SFBFF-5	P		18			18*2=36
	I		2			
Mean ± S.E.						36.6±2.2 a

Different letters represent significant difference compared with control (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
AI-SFBFF= virgin female rats injected with 100 µl of anti-inhibin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.
AA-SFBFF= virgin female rats injected with 100 µl of anti-activin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.

Table (4): Qualitative scoring of FSH IHC in adenohypophysis at diestrus phase.

Score		0	1+	2+	3+	Q = P*I
Positive Cells (P)		<10%	10-25%	25-50%	50-75%	
Score		1	2	3		
Intensity of Staining (I)		weak staining	Moderate staining	strong staining		
C-1	P		18			18*2=36
	I		2			
C-2	P		19			19*2=38
	I		2			
C-3	P		22			22*2=44
	I		2			
C-4	P		17			17*2=34
	I		2			
C-5	P		15			15*2=30
	I		2			
Mean ± S.E.						36.4±2.4 a

AI-SFBFF-1	**P**			**40**		**40*1=40**
	1	**1**				
AI-SFBFF-2	**P**		**15**			**15*2=30**
	I		**2**			
AI-SFBFF-3	**P**		**24**			**24*2=48**
	I		**2**			
AI-SFBFF-4	**P**			**28**		**28*2=56**
	I		**2**			
AI-SFBFF-5	**P**		**22**			**22*2=44**
	I		**2**			
Mean ± S.E.						**43.6±3.2 a**
AA-SFBFF-1	**P**		**15**			**15*2=30**
	I		**2**			
AA-SFBFF-2	**P**		**33**			**33*2=66**
	I		**2**			
AA-SFBFF-3	**P**		**19**			**19*1=19**
	I	**1**				
AA-SFBFF-4	**P**		**30**			**30*1=30**
	I	**1**				
AA-SFBFF-5	**P**		**24**			**24*2=48**
	I		**2**			
Mean ± S.E.						**38.6±5.4 a**

Different letters represent significant difference compared with control (P<0.05).

C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.

AI-SFBFF= virgin female rats injected with 100 µl of anti-inhibin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.

AA-SFBFF= virgin female rats injected with 100 µl of anti-activin steroid free bovine follicular fluid antiserum (ip) at proestrus phase.

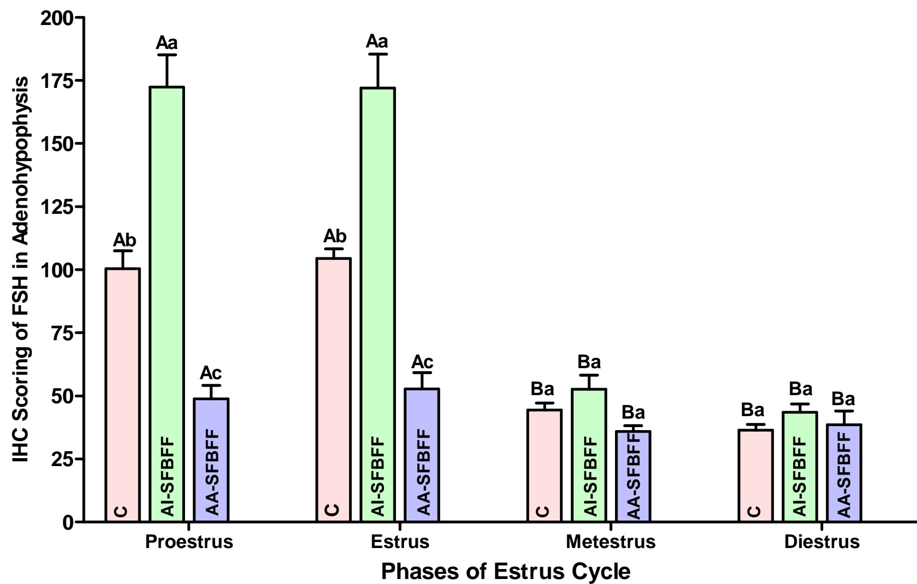

Figure (2): IHC staining scores of adenohypophysis FSH, at proestrus, estrus, metestrus and diestrus in cyclic virgin female rats treated with AI-SFBFF and AA-SFBFF.

The results presented as mean±standard error of the mean.

Different capital letters denote significant ($p<0.05$) between phases of the estrus cycle for each group.

Different small letters denote significant ($p<0.05$) between experimental groups for each phase of the estrus cycle.

C (control): cyclic virgin female rats injected with 100 µl of normal saline (ip), at early proestrus.

AI-SFBFF: cyclic virgin female rats injected with 100 µl of steroid-free BFF antiserum (ip), at early proestrus.

AA-SFBFF: cyclic virgin female rats injected with 100 µl of inhibin- and steroid-free BFF antiserum (ip), at early proestrus.