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Research article

Ovarian immunohistochemical expression of estradiol 17β in cyclic female rats treated with steroid free bovine follicular fluid antiserum

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Abstract

The current study aimed to examine the effect of steroid-free bovine follicular fluid (SFBFF) antiserum on ovarian immunohistochemical expression levels of 17β Estradiol in cyclic virgin female rats. After estrus synchronization, 80 adult virgin female Wistar rats (aged 60 days and weighed 156±4.82 g) were randomly assigned into two equal groups (40 females each) and injected intrperitoneally, at late metestrous, with 100 µl of normal saline (control) and 100 µl of SFBFF antiserum (AI-SFBFF group). At each phase of the estrus cycle, 10 females from each group were anesthetized (by injection of 0.4 ml of thiopental sod./ animal), dissected and the ovaries were obtained for determination of immunohistochemical expression density of 17β Estradiol. Ovarian examination of AI-SFBFF group females demonstrated slight increase of estradiol immunohistochemical density at proestrus and mark increase at estrus and metestrus phases. It could be concluded that immunoneutralization of endogenous inhibin by SFBFF antiserum results in high levels of estradiol actions in reproductive organs.

Keywords: Antiserum, Bovine, Estradiol, Expression, Steroid.

Introduction

are influence the reproductive activity in mammals. The antagonistic actions of activins and inhibins are important in the integration of reproductive functions, where activins stimulates FSH secretion from the adenohypophysis, the gonadal inhibin antagonizes its action by inhibiting FSH secretion, in a negative feedback mechanism (4), and therefore inhibin immunoneutralization will decrease endogenous inhibin concentrations, and subsequently discharges pituitary FSH secretion. These changes could lead to induce folliculogenesis, oocyte maturation, and oocyte number and follicular activity (5-9). Passive immunization against endogenous inhibin increased the ovulation rate via the elevated FSH secretion in rats (10). Multiple ovulations were encouraged effectively by

Rodent reproduction is regulated by accurate interactions throughout the hypothalamic-pituitary-gonadal axis, where the hypothalamus discharges matched pulses of GnRH as the primary inducer of the reproductive hormones secretion. GnRH stimulates the biosynthesis of pituitary LH and FSH that in turn promote gonadal gametogenesis and hormones secretion, as the key coordinator of the reproduction (1). LH and FSH are responsible for stimulating ovarian folliculogenesis as greater than four layers of granulosa cells as well as steroidogenesis, and ovulation (2). FSH has direct activities in the gamete production in addition to hormonal secretion (such as estradiol and inhibins) that performs feedback action on FSH secretion from the adenohypophysis (3). Activins and inhibins

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www.qu.edu.iq/journalvm

(14).The current experiment aims to investigate the role of passive immunization against inhibin, by using prepared antiserum against steroid free bovine follicular fluid on reproductive efficiency in cycling female rats, by assessment of immunohistochemical expression levels of estradiol 17β in the ovarian tissues of cycling female rats.

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 429

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 (15).

Estimation of cholesterol in charcoal treated FF:

The cholesterol has been is estimated in the FF according to Wise (15).

Preparation of steroid-free BFF antiserum:

SFBFF antiserum was used for immunization of rabbits against SFBFF (for obtaining anti-inhibin SFBFF). Five mature male rabbits have been injected with 1 ml. of 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.

Animals:

endogenous inhibin immunoneutralization in a number of species including rats (11). It has been demonstrated that immunization of different animals against inhibin has potent role in elevating the plasma FSH concentrations, enhanced follicular growth and development and finally elevated ovulation rate (10-13) and uterine implantation sites and litter size in rats

Mature virgin cycling female rats (aged 60 days and weighed 156±4.82 g) were used in the present study. They were kept under controlled condition (12L: 12D day cycles) and temperature (22-25 °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:

Eighty females were randomly assigned into two equal groups (SFBFF antiserum treated and control). At late metestrus SFBFF group females were injected with SFBFF antiserum (100 µl/ rat, ip) whereas control group females were injected with normal saline (100 µl/ rat, ip). 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 thiopental sod./ animal), dissected and the ovaries were removed and fixed in formalin 10% for immunohistochemical examination to determine the expression density of estradiol 17β.

Histological study:

Histological sections have been prepared according to Luna (1968).

Immunohistochemistry-Paraffin protocol:

According to the manufacture instructions (**www.abcam.com/technical)**, immunohistochemistry (IHC) was performed.

Statistical Analysis:

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

All values were expressed as mean± SEM. Comparisons were performed using student t-test for groups unpaired values. Differences were considered to be significant at the level of ($P<0.05$). All statistical analysis were carried out using the Graph Pad Prism (SAS Institute, Inc., USA).

Results

there is no significant changes ($p>0.05$) between experiment groups Table (4). In comparison between phases of the estrus cycle, in all experimental groups, the highest estradiol expression density has been reported at estrus and metestrus phases followed by proestrus phase, whereas the lowest densities have been reported at diestrus phase Figure (4).

Ovary estradiol IHC expression

In comparison between experimental groups, estradiol IHC expression density in ovaries of SFBFF group female rats showed mild elevation ($P>0.05$) at proestrus phase of estrus cycle Figure (1) and Table (1) and marked elevation ($P<0.05$) at both estrus phase Figure (2) and Table (2) and metestrus phases Figure (3) and Table (3) of the estrus cycle. At distrust phase of the estrus cycle,

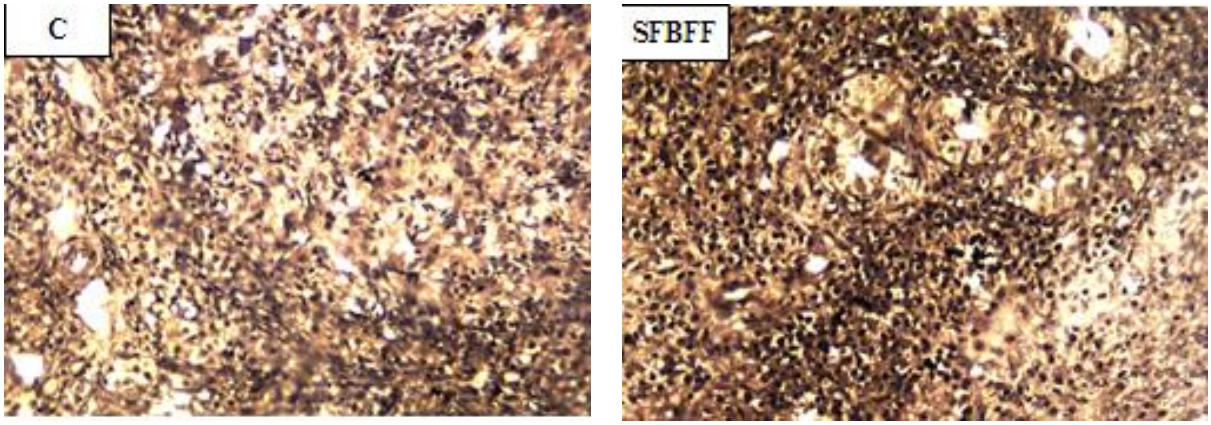

Figure (1): histological sections obtained from ovaries of control (C) and SFBFF antiserum treated female rats at proestrus phase of the estrus cycle show obvious increase in the immunohistochemical density of estradiol (brown color) in treated group compared with control. IHC stain, 100x.

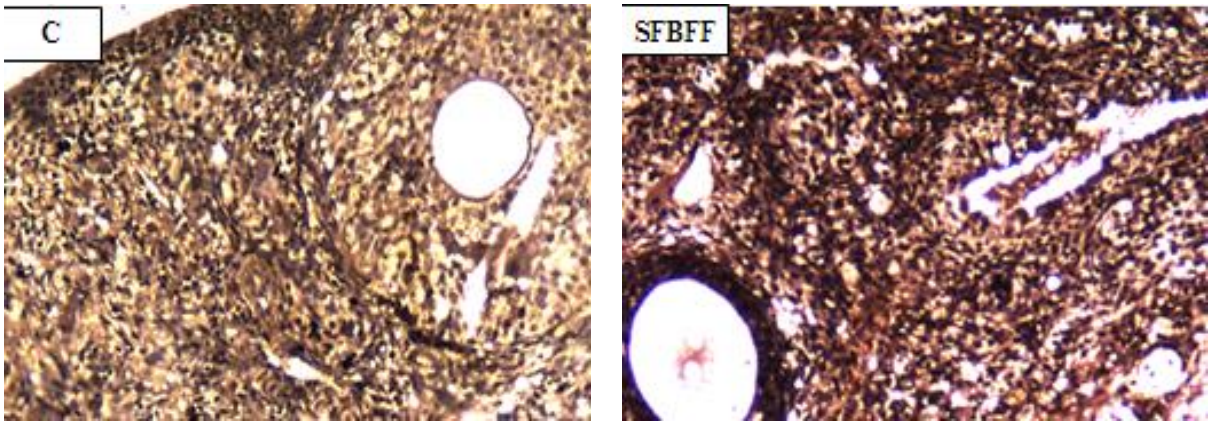

Figure (2): histological sections obtained from ovaries of control (C) and SFBFF antiserum treated female rats at estrus phase of the estrus cycle show obvious increase in the immunohistochemical density of estradiol (brown color) in SFBFF group among experimental groups. IHC stain, 100x.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

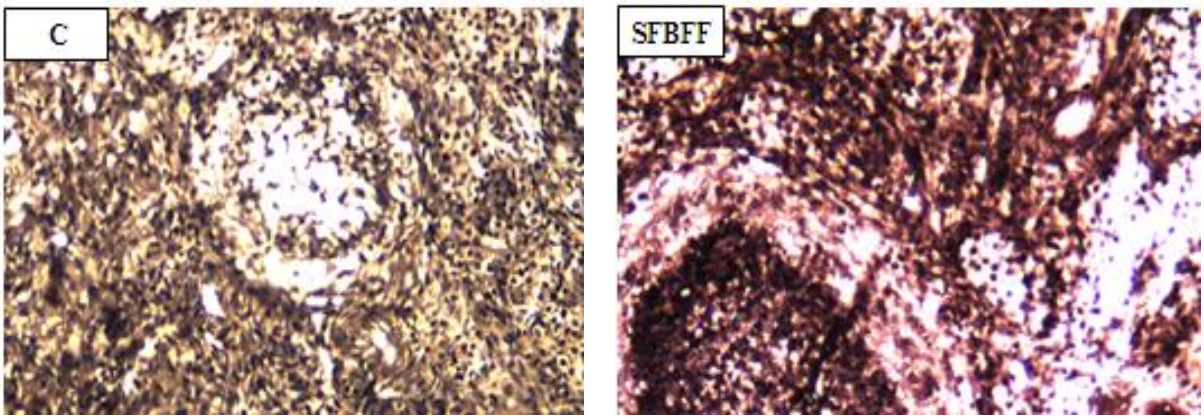

Figure (3): histological sections obtained from ovaries of control (C) and SFBFF antiserum treated female rats at metestrus phase of the estrus cycle show obvious increase in the immunohistochemical density of estradiol in SFBFF group among experimental groups. IHC stain, 100x.

Table (1): Qualitative scoring of estradiol IHC in ovaries at proestrus 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			28		28*2=56
	I		2			
C-2	P			30		30*2=60
	I		2			
C-3	P		20			20*2=40
	I		2			
C-4	P			26		26*2=52
	I		2			
C-5	P			30		30*2=60
	I		2			
Mean ± S.E.						53.6±2.7 b
SFBFF-1	P			40		40*2=80
	1		2			
SFBFF-2	P			35		35*2=70
	I		2			
SFBFF-3	P			34		34*3=102
	I				3	
SFBFF-4	P			28		28*2=56
	I		2			
SFBFF-5	P			45		45*2=90
	I		2			
Mean ± S.E.						79.6±5.3 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.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Table (2): Qualitative scoring of estradiol IHC in ovaries 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			35		35*2=70
	I		2			
C-2	P			30		30*2=60
	I		2			
C-3	P			38		38*2=76
	I		2			
C-4	P			26		26*2=52
	I		2			
C-5	P			40		40*2=80
	I		2			
Mean ± S.E.						67.6±3.4 b
SFBFF-1	P				75	75*3=225
	1				3	
SFBFF-2	P				85	85*3=255
	I				3	
SFBFF-3	P				70	70*3=210
	I				3	
SFBFF-4	P				85	85*3=255
	I				3	
SFBFF-5	P				90	90*3=270
	I				3	
Mean ± S.E.						243.0±7.8 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 (3): Qualitative scoring of estradiol IHC in ovaries 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		24			24*2=48
	I		2			
C-2	P		22			22*2=44
	I		2			
C-3	P		16			16*2=32
	I		2			
C-4	P		20			20*2=40
	I		2			
C-5	P			25		25*2=50
	I		2			
Mean ± S.E.						42.8±2.2 b
SFBFF-1	P				55	55*2=110
	1		2			
SFBFF-2	P			45		45*2=90
	I		2			
SFBFF-3	P			38		38*2=76
	I		2			
SFBFF-4	P				50	50*2=100
	I		2			
SFBFF-5	P			39		39*2=78
	I		2			
Mean ± S.E.						90.8±4.3 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.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Table (4): Qualitative scoring of estradiol IHC in ovaries 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		24			24*1=24
	I	1				
C-2	P		18			18*1=18
	I	1				
C-3	P		23			23*1=23
	I	1				
C-4	P		17			17*1=17
	I	1				
C-5	P		20			20*1=20
	I	1				
Mean ± S.E.						20.4±0.9 a
SFBFF-1	P		24			24*1=24
	1	1				
SFBFF-2	P		18			18*1=18
	I	1				
SFBFF-3	P		24			24*1=24
	I	1				
SFBFF-4	P		23			23*1=23
	I	1				
SFBFF-5	P			33		33*1=33
	I	1				
Mean ± S.E.						24.4±1.6 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.

Discussion

with control, SFBFF treated group female rats showed higher density of estradiol-17β in the ovaries at estrus phase and continued at early metestrus. This marked increment of estradiol occurred in concomitant with the notable growth of a large number of ovarian follicles in SFBFF antiserum treated females, as it has been found that passive immunization of female rats against inhibin alpha subunit increased folliculogenesis as well as Graafian and total follicle number (11). These observations clearly indicate that immunoneutralization of endogenous inhibin enhances biosynthesis of estrogens from ovarian follicles, where the high level of endogenous FSH could stimulates the wave of follicular development and results in production of a large amount of estradiol-17β, which induces the LH surge by positive feedback effect to the hypothalamus and pituitary axis, leading to induction of

From previous observations, SFBFF antiserum treated female rats reported increased immunohistochemical expression levels of pituitary FSH, early at proestrus phase and continued at estrus phase of the estrus cycle (9), whereas the increment of ovarian estradiol 17β expression levels, in the current study, was observed obviously at estrus and early metestrus phases of the estrus cycle. Meaning while the immunoneutralization against endogenous inhibin, by infusion of SFBFF antiserum, caused a rapid increase of pituitary FSH secretion and delayed increase of ovarian estradiol-17β production. This result was in agreement with that reported by (16), 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 (17). In comparison

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

against endogenous inhibin, at early proestrus, increases biosynthesis of pituitary FSH and estradiol production in the ovaries. This hormonal increment could be benefit for improvement of female reproductive fecundity, which may play a significant potent role in animal reproduction applications.

superovulation (18). In conclusion, the prepared antisera against inhibin (from steroid-free bovine follicular fluid) or activin (from inhibin and steroid-free bovine follicular fluid) were efficient in immunoneutralization of endogenous inhibin or activin. From the present observations, it can be postulated that passive immunization

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