EFFECT OF STEROID-FREE BOVINE FOLLICULAR FLUID ANTISERUM ON OVARIAN IMMUNOHISTOCHEMICAL EXPRESSION OF GH IN MATURE FEMALE RATS

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ABSTRACT

This study aims to investigate the role of steroid-free bovine follicular fluid (SFBFF) antiserum on ovarian immunohistochemical expression density of growth hormone in cyclic virgin female rats. Bovine follicular fluid was aspirated from large Graffian follicles (>15 mm in diameter), centrifuged and treated with activated charcoal. SFBFF was injected to adult male rabbits (5 injections with a week interval) for obtaining anti-inhibin SFBFF. A month after last injection, blood was collected, centrifuged and antiserum was obtained. After estrus synchronization, 80 virgin female Wistar rats (aged 58 days and weighted 150±4.5 g) were randomly assigned to two groups (40 females each) and injected intraperitonealy, at late metestrous, with 100 µl of normal saline (control) and 100 µl of SFBFF antiserum (treated group). Estrus cycle phases were monitored using vaginal smears. At each phase of the cycle, 10 females from each group were sacrificed, dissected and the ovaries were obtained for immunohistochemical expression density of GH. The results demonstrated positive immunostaining of GH at all stages of the estrus cycle with differences between groups, where treated group showed higher expression levels of GH, at both proestrus and estrus phases, among experimental groups. It can be concluded that passive immunization against inhibin by SFBFF antiserum can increase the expression density of GH in the ovaries of cyclic female rats, and could be efficient as augmented factors for reproductive potency.

INTRODUCTION

Pituitary somatotropin, also known as growth hormone (GH), has fundamental impact in somatic growth during postnatal period. GH performs its action by stimulating the

biosynthesis of hepatic and other local tissues insulin growth factors (1). In female reproduction, GH act to increase the development of ovarian follicles through its direct action on GH-receptors and indirect action by enhancing biosynthesis of hepatic and/or ovarian

IGF-1 (2). Various research observations mentioned to the decline of the number of ovarian follicles in GHR null mice (3;4), suggesting that endogenous GH has an important role in the development of ovarian follicles in the rodent thus enhancing reproductive efficiency.

Inhibin, a gonadal derived hormone, is essential for the maintenance of normal mammalian reproductive function based on its ability to inhibit FSH synthesis and release in anterior pituitary gonadotropes (5;6). Inhibin is also an autocrine/paracrine mediator within the gonads in the stimulation of thecal cell steroid production and the inhibition of oocyte maturation (7). Studies in mammals have shown that activins and inhibins are involved in many physiological processes, including reproduction. Within the reproductive system, activins and inhibins have been found to regulate the release of GnRH from the hypothalamus (8) and FSH from the pituitary (9;10). In addition, activins and inhibins exert many regulatory effects on the testis and ovary, such as steroidogenesis, proliferation of spermatogonia, proliferation of granulosa cells, modulation of FSH receptors, follicle development, and maturation. In most biological systems tested, activin and inhibin have opposite effects (10). Inhibins suggested role to date is to inhibit FSH synthesis and secretion by the pituitary (11;12). Their role is inhibitory to suppress the stimulatory action of the structurally related activins (13).

Many researchers have looked at the effect of follicular fluid. Henderson and Franchimont (14) showed that bovine follicular fluid contains inhibin, as a product of granulosa cells. Injection of bovine Follicular fluid suppressed FSH secretion from adenohypophesis in ewes and delayed the occurrence of estrous and ovulation in cows (15). On the other hand, treatment of ewes with steroid-free ovine follicular fluid prevented follicular growth (16) and decreased the secretion of ovarian estradiol as well as inhibin (17).

In vitro immunoneutralization of endogenous inhibin revealed significant increase of serum FSH and estradiol in parallel with significant decrease of inhibin and increased ovulation rate (18;19;20). Thus, immunization of animals against their endogenous inhibin by SFBFF antiserum could inspected to decreaseing the negative feedback effects of inhibin on FSH secretion, since both FSH production and serum concentrations of FSH could be increased in the immunized animals. Therefore the present study aims to examine the role of immunoneutralization against circulating inhibin by injection of prepared steroid-free BFF

antiserum on ovarian immunohistochemical expression levels of GH, as an augmented hormone for folliculogenesis.

MATERIALS AND METHODS

Collection and preparation of follicular fluid (FF)

Follicular fluid has been collected from bovine ovarian follicles (≤15mm in diameter) by aspiration. BFF were centrifuged at 8000 rpm for 15 minute at 4˚C to remove cellular debris, thereafter mixed with activated charcoal (10mg/ ml) for 1 hour at 4˚C. Charcoal was removed by centrifugation at 14000 rpm for 90 minute at 4˚C. SFBFF was kept at -20˚C until use.

Detection of proteins and cholesterol in follicular fluid: Biuret assay and ninhydrin reaction was used to detect the proteins in the FF, whereas cholesterol was estimated in the FF (Wise, 21).

Preparation of steroid-free BFF antiserum: SFBFF antiserum was used for immunization of adult male rabbits against SFBFF (for obtaining anti-inhibin SFBFF). Five male rabbits were 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.

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 58 days and weighted 150±4.5 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. Eighty female rats were randomly assigned to two equal groups (control and treatment). At late metestrous, female rats were intraperitonealy injected with 100 µl of normal saline (Control group) and 100 µl of SFBFF antiserum (treatment 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 thiopental sod./ animal), dissected and the ovaries were removed and fixed in formalin 10% for immunohistochemical examination to determine the expression density of GH.

Histological study: histological sections have been prepared according to Luna (22).

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 between experimental groups were performed using student t-test. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA).

RESULTS

The result demonstrated in figure (2) reveals the effect of treatment with steroid free bovine follicular fluid antiserum on qualitative scoring of immunohistochemical expression of GH in ovarian tissues at different stages of the estrus cycle in cyclic virgin female rats. At proestrus (figure 1-TP and table 1) and estrus (figure 1-TE and table 2) phases of the estrus cycle, the result revealed that treated female ovaries registered higher scores ($p<0.05$) of positive cells and staining intensity than control females. While metestrus (figure 1-TM and table 3) and diestrus (figure 1-TD and table 4) phases registered no significant differences ($p>0.05$) between experimental groups.

DISCUSSION

Previous studies postulated that neutralization of endogenous inhibin results in high secretion of pituitary GH (23) as well as pituitary FSH and gonadal estradiol and activin (24), which also associated with high expression levels of gonadal GH receptors (25;26;27). Also it has been found that elevated FSH levels, due to the immunoneutralization of endogenous inhibin, the dominant follicles can be survived by the local action of activin, which has been reported to induce FSH receptor mRNA expression (28) and increase its number (28;29). On the other hand, FSH increases activin receptor number in these cells (30). with high level of FSH, activin accelerates aromatase activity (31). These results indicate that activin can play a role in granulosa cell differentiation. These data demonstrate that activin stimulates the action of gonadotropins at the level of the ovary and enhances follicular development.

The GHR is expressed in the ovary of many different species, including rats (26). The GHR is concentrated in the membrana granulosa of primary and small to medium sized antral follicles

and in corpus luteum CL. In the CL, GHR messenger RNA (mRNA) and immunoreactivity are concentrated in the production large luteal cells responsible for high-level progesterone production (32). GH does not seem to have an essential role in CL development or function in mice, however, because implantation and gestation occur normally in the GHR null mice.

GH was found to be necessary for the sexual maturation, since absent or delayed puberty in relation to GH deficiency can be modulated by GH administration (33;34). In the present study, it has been reported that ovarian IHC expression of GH increased by infusion of SFBFF antiserum. This increment may reflected in modulation of ovarian steroidogenesis, gametogenesis and gonadal differentiation as well as gonadotrophin secretion and responsiveness.

The increment of the ovarian growth factors, namely activin, due to passive immunization against inhibin-α subunit, may also mediate the actions of GH, since follistatin (which binds and inactivates activin) blocks the stimulatory effect of GH on pre-antral follicle growth (25). GH may thus be particularly important in the recruitment of follicles and initiation of oocyte growth.

The increment of ovarian GH 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, since both GH and gonadotrophins are required to prevent atresia of larger follicles (>2 mm) following hypophysectomy in sheep (35). GH may play a role in the development of the dominant follicle (36). The stimulatory effect of GH on follicle number and size reflects increased cell proliferation (37), and also is indicative of the suppressive effect of GH on apoptosis (38;39;40).

On the other hand, the present findings could be attributed to the increment of serum estrogen level after infusion of inhibin antibody (27), where this increment was previously postulated by Al-Sa'aidi and Thanoon to be positively correlated to the up-regulation of hypothalamic *GHRH* and pituitary *GH* genes, as GH has been shown to affect oestrogen synthesis both *in vivo* and *in vitro*, since steroidogenic action of GH is associated with increased activity of several enzymes and may be partially responsible for the facilitator effect of GH on folliculogenesis and gametogenesis (41). GH enhances aromatase and 3-β-hydroxysteroid dehydrogenase activity (42), and therefore GH enhances oestradiol secretion from granulosa cells (37;43;44). Other studies indicate that GH and gonadotrophins act synergistically to increase oestradiol synthesis in granulosa cells (45;46). This synergy may reflect upregulation

of gonadotrophin receptors by GH or the upregulation of GHRs by gonadotrophin induced cAMP (47).

It can be concluded that passive immunization against inhibin by SFBFF antiserum can increase localization density of GH in the ovaries of cyclic female rats, and could be acted as augmented factors for reproductive potency.

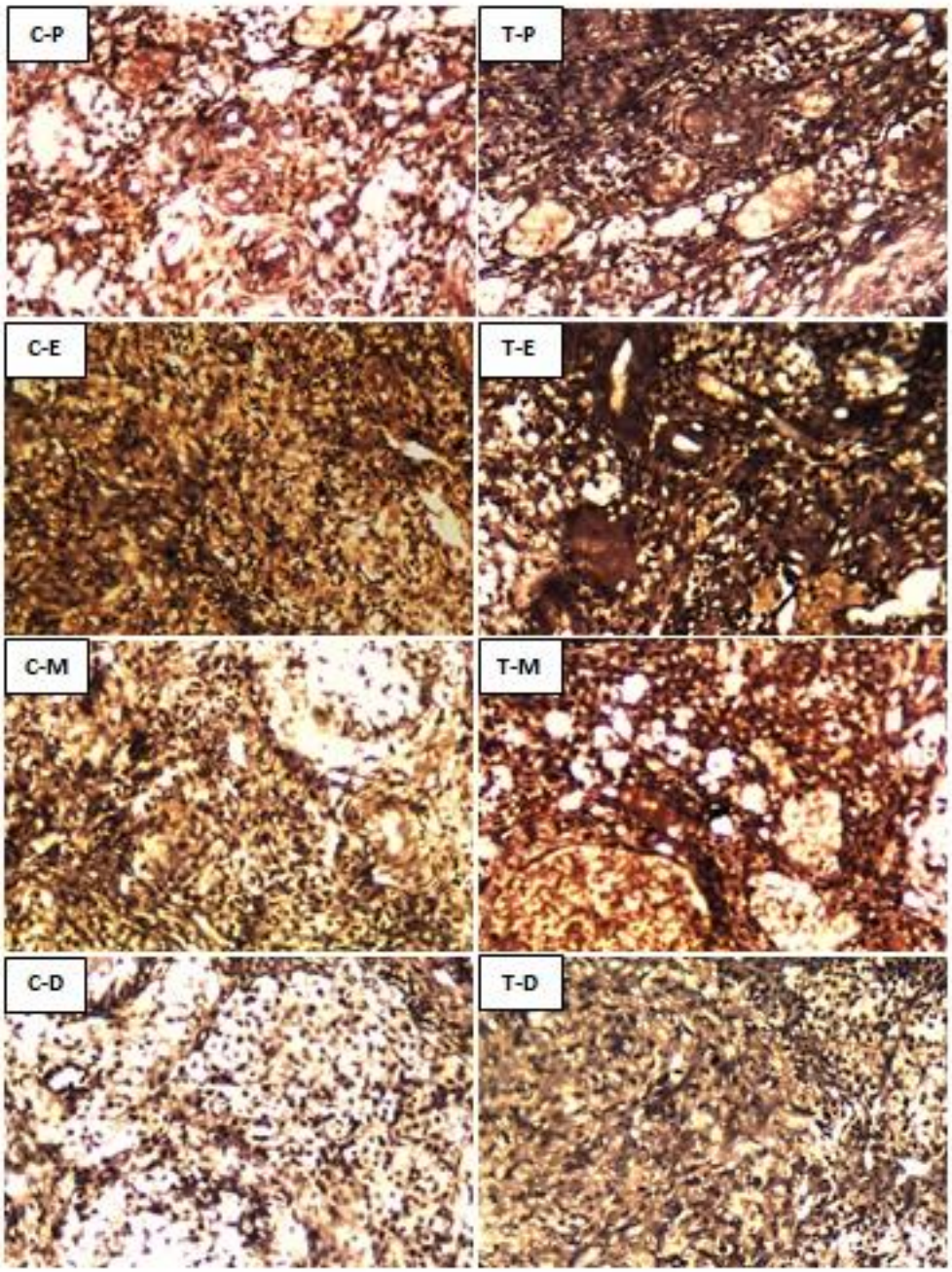

Figure (1): Ovarian IHC expression of GH in control (C) and SFBFF antiserum (T) cyclic female rats at estrus cycle phases. High expression of was shown in treated females at proestrus (T-P) and estrus (T-E) comared with the corresponding phases in control (C-P and C-E), whereas metestrus (C-M and T-M) and diestrus (C-D and T-D) show insignificant differences in their densities. IHC x200.

Table (1): Qualitative IHC scoring of ovarian GH 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 stainin	strong staining		
C-1	P				55	55*2=110
	I		2			
C-2	P			35		35*2=70
	I		2			
C-3	P				50	50*2=100
	I		2			
C-4	P				51	51*2=102
	I		2			
C-5	P			43		43*2=86
	I		2			
C-6	P			47		47*2=94
	1		2			
C-7	P				55	55*2=110
	I		2			
C-8	P			40		40*3=120
	I			3		
C-9	P			45		45*2=90
	I		2			
C-10	P				52	52*2=104
	I		2			
Mean ± S.D.						106.1±23.91 b
T-1	P				55	55*2=165
	I			3		
T-2	P				55	55*2=110
	I		2			
T-3	P			45		45*3=135
	I			3		
T-4	P				50	50*3=150
	I			3		
T-5	P			45		45*2=90
	I		2			
T-6	P				60	60*2=120
	I		2			
T-7	P				65	65*2=130
	I		2			
T-8	P				55	55*3=165
	I			3		
T-9	P			45		45*2=90
	I		2			
T-10	P				55	55*3=165
	I			3		
Mean ± S.D.						132.0±29.36 a

Different letters represent significant difference between groups (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
T= virgin female rats injected with 100 µl of SFBFF antiserum (ip) at proestrus phase.

Table (2): Qualitative IHC scoring of ovarian GH 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 stainin	strong staining		
C-1	P				65	65*2=130
	I		2			
C-2	P				65	65*2=130
	I		2			
C-3	P				55	55*3=165
	I			3		
C-4	P				70	70*2=140
	I		2			
C-5	P				65	65*2=130
	I		2			
C-6	P				60	60*3=180
	1			3		
C-7	P				65	65*2=130
	I		2			
C-8	P				70	70*2=140
	I		2			
C-9	P				80	80*2=160
	I		2			
C-10	P				50	50*3=150
	I			3		
Mean ± S.D.						145.5±17.71 b
T-1	P				70	70*2=140
	I		2			
T-2	P				75	75*3=225
	I			3		
T-3	P			70		70*3=210
	I			3		
T-4	P			65		65*2=130
	I		2			
T-5	P			60		60*3=180
	I			3		
T-6	P			65		65*3=195
	I			3		
T-7	P				75	75*3=225
	I			3		
T-8	P			70		70*2=140
	I		2			
T-9	P				75	75*2=140
	I		2			
T-10	P				70	70*3=210
	I			3		
Mean ± S.D.						179.5±38.55 a

Different letters represent significant difference between groups (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
T= virgin female rats injected with 100 µl of SFBFF antiserum (ip) at proestrus phase.

Table (3): Qualitative IHC scoring of ovarian GH 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 stainin	strong staining		
C-1	P			45		45*2=90
	I		2			
C-2	P			45		45*2=90
	I		2			
C-3	P				55	55*2=110
	I		2			
C-4	P			40		40*2=80
	I		2			
C-5	P			35		35*2=70
	I		2			
C-6	P			45		45*2=90
	1		2			
C-7	P			35		35*2=70
	I		2			
C-8	P			45		45*3=90
	I		2			
C-9	P			35		35*2=70
	I		2			
C-10	P				50	50*2=100
	I		2			
Mean ± S.D.						86.0±13.49 a
T-1	P			45		45*2=90
	I		2			
T-2	P				55	55*2=110
	I		2			
T-3	P				55	55*2=110
	I		2			
T-4	P				60	60*2=120
	I		2			
T-5	P			35		35*2=70
	I		2			
T-6	P			40		40*2=80
	I		2			
T-7	P			45		45*2=90
	I		2			
T-8	P			45		45*2=90
	I		2			
T-9	P				55	55*2=110
	I		2			
T-10	P			30		30*3=60
	I		2			
Mean ± S.D.						93.0±19.47 a

Different letters represent significant difference between groups (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
T= virgin female rats injected with 100 µl of SFBFF antiserum (ip) at proestrus phase.

Table (4): Qualitative IHC scoring of ovarian GH 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 stainin	strong staining		
C-1	P			45		45*2=90
	I		2			
C-2	P			40		40*2=80
	I		2			
C-3	P			35		35*2=70
	I		2			
C-4	P			45		45*2=90
	I		2			
C-5	P			45		45*2=90
	I		2			
C-6	P			40		40*3=120
	1			3		
C-7	P			40		40*2=80
	I		2			
C-8	P			40		40*3=120
	I			3		
C-9	P			35		35*2=70
	I		2			
C-10	P			30		30*2=60
	I		2			
Mean ± S.D.						87.0±20.03 a
T-1	P			45		45*2=90
	I		2			
T-2	P				50	50*2=100
	I		2			
T-3	P			35		35*2=70
	I		2			
T-4	P			40		40*2=80
	I		2			
T-5	P			35		35*2=70
	I		2			
T-6	P			40		40*3=120
	I			3		
T-7	P			45		45*2=90
	I		2			
T-8	P			45		45*2=90
	I		2			
T-9	P			40		40*2=80
	I		2			
T-10	P				55	55*2=110
	I		2			
Mean ± S.D.						90.0±16.33 a

Different letters represent significant difference between groups (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
T= virgin female rats injected with 100 µl of SFBFF antiserum (ip) at proestrus phase.

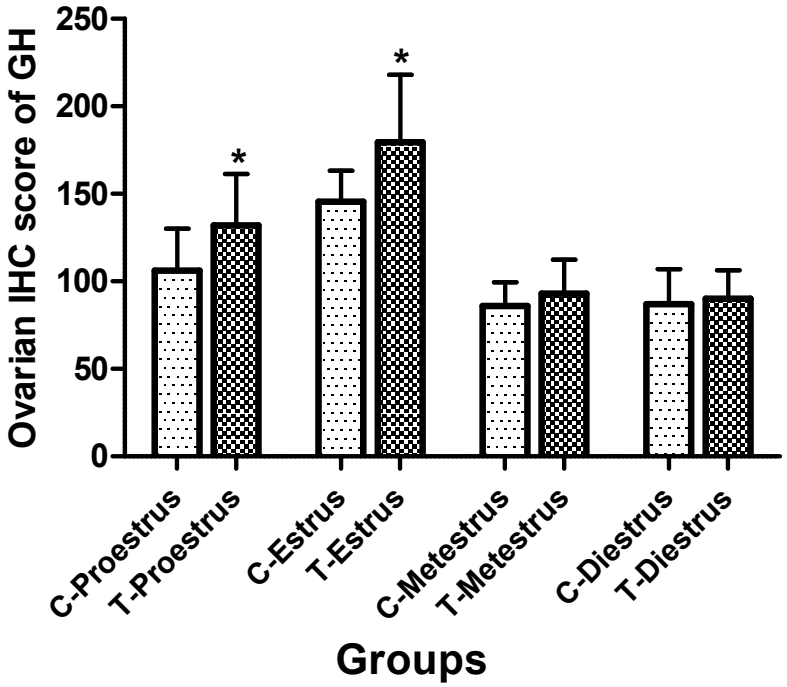

Figure (2): Quantitative ovarian IHC score of GH in cyclic female rats treated with SFBFF antiserum.

Stars denote significant difference between groups (P<0.05).
C = virgin female rats injected with 100 µl of normal saline (ip) at proestrus phase.
T= virgin female rats injected with 100 µl of SFBFF antiserum (ip) at proestrus phase.

تأثير مضاد السائل الجريبي البقري منزوع الستيرويدات على التعبير الكيميائي النسجي المناعي لهرمون النمو في مبايض الجرذان الناضجة

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الخلاصة

استهدفت الدراسة الحالية التحري عن دور المضاد المصلي للسائل الجريبي منزوع الستيرويدات على كثافة التعبير الكيميائي النسجي المناعي لهرمون النمو في مبايض الجرذان الناضجة. تم سحب السائل الجريبي من جريبات كراف ناضجة (أقطارها أكبر من 15 ملم) ودورت بجهاز الطرد المركزي ثم عوملت بالفحم المنشط. حقن السائل الجريبي المحضر في أرانب ناضجة (حقنة واحدة اسبوعيا لمدة خمسة أسابيع) لغرض الحصول على المضاد المصلي للسائل الجريبي منزوع الستيرويدات. بعد مرور شهر على آخر حقنة يم سحب الدم من الأرانب ودور بجهاز الطرد المركزي وجمع منه مصل الدم وحفظ بدرجة -20 مئوية الى حين الاستخدام. بعد توحيد الشبق، تم توزيع 80 من إناث الجرذان البالغة (بعمر 58 يوما ومعدل وزن 150 ± 4.5 غرام) عشوائيا على مجموعتين (40 لكل مجموعة) وحقنت في البريتون في نهاية طور metestrus بـ 100 مايكرلتر من المحلول الفسلجي (السيطرة) أو 100 مايكرولتر من المضاد المصلي (المعاملة). تمت مراقبة أطوار الشبق باستخدام المسحة المهبلية. تمت التضحية بعشرة اناث من كل مجموعة في كل طور

وأزيلت منها المبايض لغرض الدراسة الكيميائية النسجية المناعية لمعرفة كثافة تعبير هرمون انمو فيها. أظهرت النتائج تعبيرا ايجابيا لتعبير هرمون النمو في جميع أطوار الشبق مع وجود اختلاف بين المجموعتين إذ أظهرت اناث المعاملة تعبيرا أعلى كثافة من إناث السيطرة خلال طوري الشبق وماقبل الشبق. يمكن الاستنتاج أن التمنيع الميسر ضد الأنهبين باستخدام المضاد المصلي للسائل الجريبي منزوع الستيرويدات يزيد من كثافة تعبير هرمون النمو في مبايض إناث الجرذان الناضجة. ويمكن استخدام هذا الفعل في زيادة خصوبة الاناث التكاثرية.

الكلمات المفتاح: المبيض، السائل الجريبي، الأنهبين، هرمون النمو.

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