Adenohypophysial mRNA Expression Level of *GH* Gene and Immunohistochemical Expression of Somatotrophs in Pregnant and Lactating Inhibin-α Immunoneutralized Female Wister Rats

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Abstract

Inhibins and activins are members of TGF-β superfamily associated with reproductive processes. The role of passive immunization against endogenous inhibin-α subunit on pituitary mRNA expression level of *GH* gene and immunohistochemical expression of somatptrophs during pregnancy and lactation in primiparous female Wister rats, has been examined in this study. Forty eight pregnant females were assigned to 2 groups (24 per each). On 5^{th} and 10^{th} day of gestation, control (C) was injected with saline (100µl, *i.p.*) and treated (INHA) was injected with inhibin-α antiserum (1µg in 100µl of saline, *i.p.*). Each group was allocated to 3 equal subgroups: pregnancy, delivery, and lactation, were sacrificed on day 16 of gestation, at parturition, and 11^{th} day of lactation, respectively. At the end of each subgroup period, females were anesthetized, dissected and adenohypophysis was removed and kept at -70 C° for molecular evaluation and others kept in formaline (10%) for immunohistochemistry. Pituitary *GH* gene expression levels in INHA group increased significantly compared with control in all periods. Expression of pituitary somatotrophs showed higher number of positive stained cells and intensity of staining in INHA group during pregnancy, delivery and lactation. In comparison between periods, in both groups, higher score has been shown at delivery, whereas lowest score recorded at lactation. In conclusion, passive immunization against inhibin-α subunit at 5^{th} and 10^{th} day of pregnancy, perform potent role in GH secretion in primiparous female Wister rats.

Physiology Classification QP_1 -345

Key words: passive immunization, inhibin, activin, TGF-β, GH, pituitary gland.

PRL secretion (6). E2 and GH or IGF-1 might be multileveled with synergy between GH and E2 occuring in the stromal compartment and synergy between IGF-1 and E2 in the epithelial compartment, both by different mechanism. In this regard IGF-1 has been shown to activate ER transcription in mammary and pituitary tumor cells (7). GH induce ER in the stromal compartment of the mammary gland. Although GH is not required for alveologenesis development, but its lactogenic function in PRLR null mammary gland has been demonstrated (4). Although growth hormone (GH) has long been known to contribute to ductal and lobuloalveolar growth this effect was attributed to structural similarities between GH and PRL, enabling GH bind to and activate PRL receptors (8). GH was shown to be more potent mammogen than PRL (9), as in PRL. deficient lactating rate, GH was found to maintain the levels and activity of mammary acetyl-CoA carboxylase and lipoprotein lipase suggesting that GH regulate the fat content of the milk (10). More studies indicate that GH is essential for mammary development in rat (11). In lactating rats, treatment with an antiserum against rGH reduced milk yield by 50% (12). It appears that GH may influence ovarian follicle development both through enhancing circulating and local ovarian IGF-1, and by direct, GH receptor (GHR)–mediated effects in the ovary (13). IGF-1 infusion did not rescue fertility or ability to respond to

Introduction:

During pregnancy, a surge of hormones result in major structural changes in the mammary gland (1). The branched ductal system, that expanded during puberity, a further develop into lobuloalveolar compartment, progesterone receptor direct lobuloalveolar development during pregnancy (2). All phases of functional differentiation occur during pregnancy which start by proliferative phase (ductal and alveolar proliferation) in early pregnancy, secretory activation in mid pregnancy and part of secretory activation phase at around parturition and contine during lactation (3). Growth hormone is the third major influence in the pubertal mammary gland and its receptor (GHR). Knockout (KO) studies have shown that GHR is required in mammary stroma for successful ductal outgrowth and side-branching (4). GHR also functions in a paracrine way, by increasing local IGF-1 expression in the mammary gland which, in synergy with estrogen, induces proliferation in neighboring epithelial cells (5). The role of growth hormone (GH) and EGF receptor in mammary gland was examined by Gallego *et al.* (4).

Estrogen have a stimulatory role on growth hormone (GH) since administration of an anti-estrogen also resulted in a fall in GH concentration in spite of reduced prolactin

somatotropic cells during pregnancy, delivery, and lactation.

Materials and methods

Preparation of Inhibin-α subunit antiserum 1%: Inhibin-α antiserum (1µg/100µl) were prepared according to the manufacture instructions (ABO, Switzerland).

Experimental animals: Sixty five days old mature primiparous female Wister rats, born at the animal house of the College of Veterinary Medicine, Basrah University, and reared under controlled conditions (12 L:12 D cycles and ambient temperature at 22 ± 2 °C) and fed on standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water *ad libitum*. Female rats were allowed to mate with experienced males (1 male with 2 females). The appearance of vaginal plug was considered as the first day of pregnancy. Forty eight pregnant females were randomly assigned to 2 groups (24 per each). On 5^{th} and 10^{th} day of gestation, control (C) was injected with saline (100µl, *i.p.*) and treated (INHA) was injected with inhibin-α antiserum (1µg in 100µl of saline, *i.p.*). Each group was allocated to 3 equal subgroups (8 females each): pregnancy, delivery, and lactation, were sacrificed on day 16 of gestation, at parturition, and 11^{th} day of lactation, respectively. At the end of each treatment and control subgroups period, female rats were anesthetized (by injection of 0.3 ml ketamine + 0.1 ml xylazine/kg body

gonadotropin in GHR null mice (14). These observations suggest that endogenous GH enhances the recruitment or survival of follicles in the ovary (perhaps independent of IGF-1 effects) thus enhancing reproductive potential (15).

Inhibins and activins are members of the TGF-β superfamily of extracellular signaling molecules (16). These members have been implicated as autocrine and paracrine regulators of ovarian follicle development and survival. Activin stimulates whereas inhibin blocks follicle-stimulating hormone biosynthesis and secretion from pituitary gonadotrope cells, and together, inhibin and activin control the pituitary gonadal axis essential for normal ovarian function (17,18). Immunoneutralization of endogenous inhibin was thought to result in diminished negative feedback on the anterior pituitary resulting in increased secretion of FSH, greater follicular development and increased ovulation rate (19,20) and puberty in immature female rats (21,22). Early onset of puberty (time of first ovulation) was also observed after immunization early with an inhibin-enriched bovine follicular fluid preparation (23).

The present study aims to investigate the role of passive immunization against endogenous circulating inhibin as early in the first trimester of pregnancy of female rats on pituitary expression level of *GH* gene and immunohistochemical expression of

significant at the level of $P<0.05$. All statistical analysis were carried out using the GraphPad Prism-5.

Results

Molecular analysis:

Quantitative Reverse Transcriptase Real-Time PCR: Data analysis of SYBR®green based reverse transcriptase RT-PCR assay were divided into primer efficiency estimation and relative quantification of *GH* gene expression levels normalized by housekeeping gene expression (*GapdH*).

Primer efficiency estimation: The data result, threshold cycle numbers (Ct) were calculated from amplification plot of real-time PCR detection system, during exponential phase of fluorescent signals of SYBR®green primer of different genes that react with complementary DNA (cDNA) of rat pituitary mRNA, where, the amount of PCR product (DNA copy numbers) in master mix reaction is approximately doubles in each PCR cycle. First prepared series dilution of pituitary cDNA of control group, this concentrations was used with the primer of *GH* gene to formation the amplification plot of each gene and then from this amplification plot threshold cycle (Ct) was used to calculate a linear regression based on the data points, and inferring the efficiency of the primer from the slope of the line.

Relative quantification of target gene expression: To calculate the relative expression of target gene in pituitary gland, weight, *i.p.*) (24), dissected and anterior pituitary was removed and kept at -70 Cº for evaluation of mRNA expression level of *GAPDH* gene as Housekeeping gene and *GH* gene by using quantitative reverse transcriptase Real-Time PCR technique based on Syber Green dye. Other pituitaries have been reserved in the formalin (10%) for histophysiological and immunohistochemical study.

Quantitative Reverse Transcriptase Real-Time PCR: qRT-PCR technique was used for quantification of *GH* gene expression levels relative to Housekeeping gene *GAPDH* gene in the pituitary. This technique was done according to method described by Wang and Hardy (13).

Immunohistochemistry-Paraffin protocol: According to Luna (25), histological sections have been prepared from pituitaries. According to the manufacture instructions (Abcam, UK; **www.abcam.com/technical)**, immunohistochemistry has been performed for demonstrating the presence and location of GH in somatotrpic cells of the pituitaries in tissue sections.

Statistical Analysis: Mean and standard error of the variables included in the present study has been calculated for each group. Student's *t*-test has been performed to test the effect of treatment in each period and one way analysis of variance (ANOVA1) and newman- keuls has been performed to test the effect of periods. Differences were considered to be

Relative quantification of pituitary *GH* gene expression: Figure (1) demonstrates the levels of GH gene expression in the pituitary obtained from treated female rats during pregnancy, delivery, and lactation periods. The expression level during pregnancy increased significantly ($p<0.05$) in INHA (8.07 folds). During delivery period, INHA group recorded significant ($p<0.05$) elevation of pituitary GH gene expression levels (12.29 folds). Same results has been registered during lactation period, where INHA recorded higher fold changes (6.72 folds) than control ($p<0.05$).

the $2^{-\Delta\Delta Ct}$ livak and Schmittgen method has been used by normalize gene expression of target gene with expression of housekeeping gene (*GAPDH*) as reference gene. The gene expression in control was expressed as (calibrator) or control in both target gene and reference gene (*GAPDH*), at first, the threshold cycle number of target gene normalized to that of reference gene in treatment group and calibrator. Second, the ΔCt of treatment group normalized to the ΔCt of calibrator, and finally the expression ratio (fold change) was calculated. In all periods, fold changes were normalized according to control (which is equal to 1).

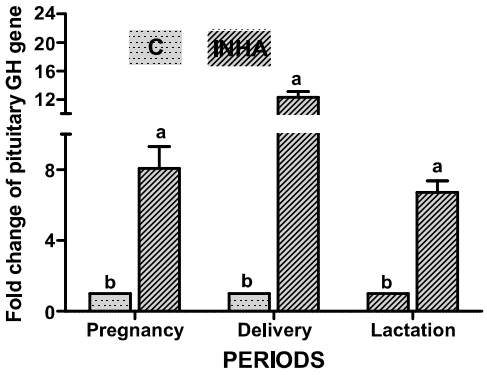

Figure (1): Effect of passive immunization against inhibin-α subunit on pituitary GH gene expression level (fold changes) during pregnancy, delivery, and lactation in pregnant rats.
Values represents mean±standard error.
Different small letters represents significancy ($p<0.05$) in comparison between groups.
C: pregnant rats injected with normal saline (100 µl, *ip*) on 5th and 10th day of pregnancy.
INHA: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

after parturition (figure 4), and the 11th day of lactation (figure 5). Quantitative scoring results of pituitary somatotrophic cells in INHA group at the 16th day of pregnancy (table 1), at the 1st day after parturition (table 2), and at mid lactation (table 3) recorded

Immunohistochemical analysis

The somatotrophic cells have been detected in normal pregnant rats anterior pituitary (control) and passive immunized pregnant rats against inhibin-α subunit (INHA group) using IHC staining technique, at the 16th day of pregnancy (figure 3), the 1st day

control (figure 2). significant increase ($p<0.05$) compared with

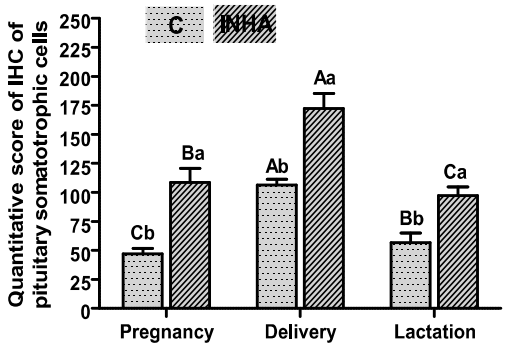

Figure (2): Effect of passive immunization against inhibin-α subunit on quantitative score of IHC of pituitary somatotrophic cells during pregnancy, delivery, and lactation in pregnant female rats.

Values represents mean±standard error.

Different small letters represents significancy ($p<0.05$) in comparison between groups.

Different capital letters represents significancy ($p<0.05$) in comparison between periods.

C: pregnant rats injected with normal saline (100 µl, *ip*) on 5th and 10th day of pregnancy.

INHA: pregnant rats injected with inhibin- α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

Table (1): Qualitative scoring of IHC of pituitary somatotrophic cells at the 16th day of pregnancy.

Score		0	1+	2+	3+	Q = P*I
Positive Cells (P)		<10%	10-25%	25-50%	50-75%	
Score		1	2	3		
Staining Intensity (I)		weak	Moderate	strong		
C-1	P		20			20*2=40
	I		2			
C-2	P			28		28*2=56
	I		2			
C-3	P			35		35*1=35
	I	1				
C-4	P			30		30*2=60
	I		2			
C-5	P		22			22*2=44
	I		2			
Mean ± S.E.						47.0±4.71 b
INHA-1	P			43		43*2=86
	I		2			
INHA-2	P			46		46*2=92
	I		2			
INHA-3	P				51	51*3=153
	I			3		
INHA-4	P				58	58*2=116
	I		2			
INHA-5	P			48		48*2=96
	I		2			
Mean ± S.E.						108.6±12.11 a

Values represents mean±standard error.
Different small letters represents significancy ($p<0.05$) in comparison between groups.
C: pregnant rats injected with normal saline (100 µl, *ip*) on 5th and 10th day of pregnancy.
INHA: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

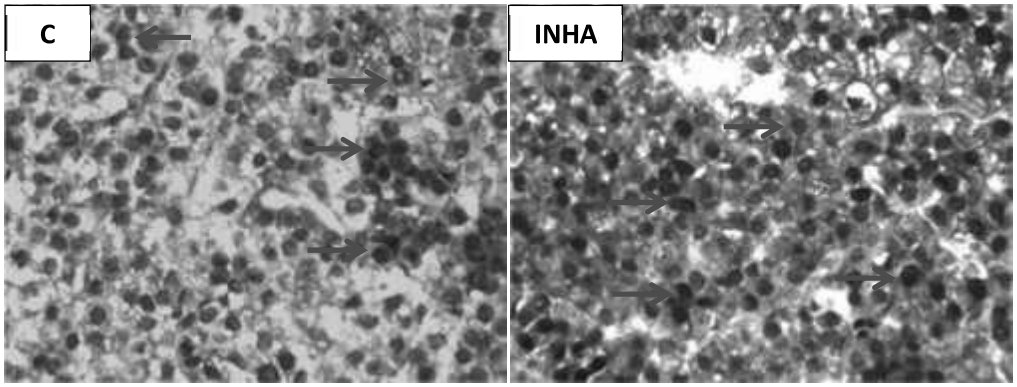

Figure (2) Pituitary from control (C) and inhibin-α (INHA) antisera injected female rats at the 16th day of pregnancy reveals actively staining of somatotrophic cells with immunohistochemistry (red arrows). Immunohistochemistry, 500X.

Table (2): Qualitative scoring of IHC of pituitary somatotrophic cells at the 1st day after parturition.

Score		0	1+	2+	3+	Q = P*I
Positive Cells (P)		<10%	10-25%	25-50%	50-75%	
Score		1	2	3		
Staining Intensity (I)		weak	Moderate	strong		
C-1	P				50	55*2=110
	I		2			
C-2	P			45		45*2=90
	I		2			
C-3	P				55	55*2=110
	I		2			
C-4	P				51	51*2=102
	I		2			
C-5	P				60	60*2=120
	I		2			
Mean ± S.E.						106.4±4.96 b
INHA-1	P				70	70*3=210
	I			3		
INHA-2	P				55	55*3=165
	I			3		
INHA-3	P			2		74*2=148
	I			74		
INHA-4	P				3	48*3=144
	I		48			
INHA-5	P				3	65*3=195
	I			65		
Mean ± S.E.						172.4±12.29 a

Values represents mean±standard error.
Different small letters represents significancy ($p<0.05$) in comparison between groups.
C: pregnant rats injected with normal saline (100 µl, *ip*) on 5th and 10th day of pregnancy.
INHA: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

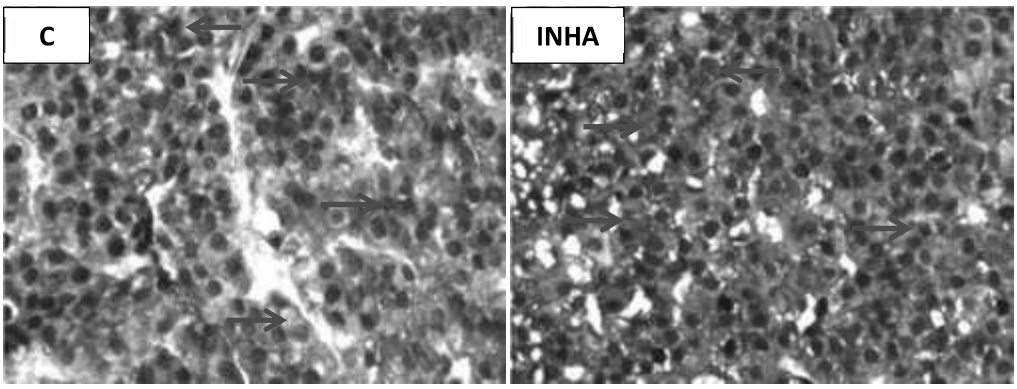

Figure (3) Pituitary from control (C) and inhibin-α (INHA) antisera injected female rats at the 1st day after parturition reveals actively staining of somatotrophic cells with immunohistochemistry (red arrows). Immunohistochemistry, 500X.

Table (3): Qualitative scoring of IHC of pituitary somatotrophic cells at the 11th day of lactation.

Score		0	1+	2+	3+	Q = P*I
Positive Cells (P)		<10%	10-25%	25-50%	50-75%	
Score		1	2	3		
Staining Intensity (I)		weak	Moderate	strong		
C-1	P	25				25*2=50
	I	2				
C-2	P	20				20*2=40
	I	2				
C-3	P	22				22*2=44
	I	2				
C-4	P	33				33*2=66
	I	2				
C-5	P	42				42*2=84
	I	2				
Mean ± S.E.						56.8±8.06 b
INHA-1	P	45				45*2=90
	I	2				
INHA-2	P	60				60*2=120
	I	2				
INHA-3	P	35				35*3=105
	I	3				
INHA-4	P	48				48*2=96
	I	2				
INHA-5	P	25				25*3=75
	I	3				
Mean ± S.E.						97.2±7.46 a

Values represents mean±standard error.
Different small letters represents significancy ($p<0.05$) in comparison between groups.
C: pregnant rats injected with normal saline (100 µl, *ip*) on 5th and 10th day of pregnancy.
INHA: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

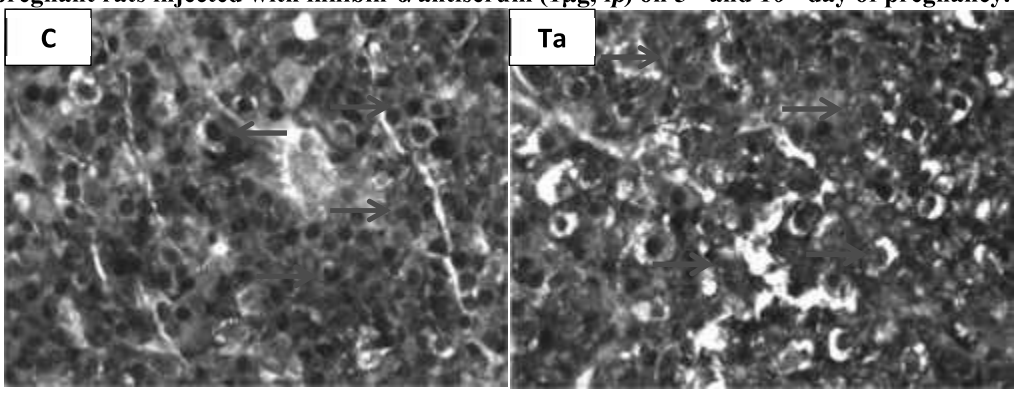

Figure (4) Pituitary from control (C) and inhibin-α (INHA) antisera injected female rats at the 11th day of lactation reveals actively staining of somatotrophic cells with immunohistochemistry (red arrows). Immunohistochemistry, 500X.

Discussion

regulatory functions at different stages of gland development. During pregnancy considered as a period of allometric growth, keeping up with overall body development, until puberty when expansive proliferation occurs, filling the fat pad under the influence of hormones and growth factors (29).

Studies demonstrated that extracts of the pituitary gland regulate mammary gland function, with researchers observing enhanced mammogenesis and lactogenesis upon its administration (30). The identified hormones that are responsible for these effects: growth hormone and prolactin. Later studies showed that mammary pubertal development was disrupted in mice lacking *Gh*, insulin-like growth factor 1 (*Igf1*) or estrogen receptor (α) (*Esr1*), genes that mediate pathways regulating ductal outgrowth and morphogenesis. In contrast, development of the adolescent gland occurs normally in mice lacking *Prl* or progesterone receptor (*Pgr*), genes that mediate signaling pathways regulating alveologenesis. So growth hormone is an important global regulator of mammary gland development, but its effect on the mammary gland may mediated largely, or even entirely, through IGF1 whose production it stimulates in the stroma (31).

In INHA group, the changes could related to the increase in the level of activins, where the immunization against inhibin-α subunit sharply decreased the concentration of inhibins (both inhibin-A and inhibin-B), this

During pregnancy, the significant high expression level of *GH* gene, recorded in INHA group compared with control, could attributed to the decrease in inhibins, as it has been reported by Thanoon (26) that passive immunization of neonate female rats against inhibin-α subunit increased expression levels of hypothalamic GHRH and pituitary GH genes. Immunization against α subunit could neutralized both types of inhibins (A and B). At delivery, both groups recorded significant elevation compared with their corresponding levels at pregnancy, with the remaining of differences in INHA compared with control. During this period, the elevation of GH may attributed to the increase in serum estradiol secretion at the end of gestation (27). At mid lactation, the level of GH decreased sharply in both groups, which may also attributed to the decrease of estradiol (26, 28).

INHA group, at all experimental period, revealed higher levels of GH which may attributed to the increase of activins and estradiol levels after immunoneutralization against inhibin-α subunit, since subsequent stages of development (pubertal growth, pregnancy, lactation, and involution) occur postnatally under the regulation of hormones. Puberty initiates branching morphogenesis, which requires growth hormone (GH) and estrogen, as well as insulin-like growth factor 1 (IGF1), to create a ductal tree that fills the fat pad. These processes require numerous signaling pathways that have distinct

cells (37), as estrogen stimulate mammary ductal growth and this stimulation is blocked by estrogen receptor antagonist, tamoxifen (38). This finding is in agreement with our result, in regard to the high *GH* gene expression in the pituitary gland in INHA group during pregnancy, delivery, and mid lactation. Therefore, it can be suggested that activin-A increase during pregnancy has a significant role in increasing *GH* gene expression level. At delivery and mid lactation, the increase in *GH* gene expression level not related to activin-A role.

Somatotrophic cells have been detected in the anterior pituitary glands of the experimental groups, using immunohistochemical staining technique, to qualify the quantitative scoring (number of positive stained cells and intensity of staining) differences between normal and inhibin-α immunoneutralized pregnant rats. The present findings, in regards to the significant elevation of quantitative scoring of somatotrophic cells in INHA group, could attributed to the decrease in the concentrations of inhibins (namely inhibin-B) together with the increase of activins (namely activin-A) during pregnancy and at delivery. These event was in orchestration with the result of pituitary *GH* gene expression levels, reported in the present study. This result is in agreement with that reported by Thanoon (26). At mid lactation, the quantitative scoring of somatotrophic cells decreased in will allow activins (namely activin-A) to perform its action, as it has been mentioned that inhibins and activins antagonistic to each other and compete on the activin receptors (32). It has been mentioned that activin-A become detactable and increased in the last half of pregnancy, where it is the predominant growth factor during mid to late pregnancy (33). On the other hand, it has been reported, at delivery and mid lactation in the present study, that immunoneutralization against inhibin-α subunit increased the functional activity of pituitary gland in protein synthesis which was over that recorded in control. This increase during pregnancy could attributed to the increase activin-B level which required for branching and alveologenesis (34), as activin enhance aromataes activity and estradiol production (35), so it enhance side branching in the present of estrogen and progesterone.

At delivery and mid lactation, the increment not related to activin-A secretion, because of expulsion of placenta, which is the main source of activin-A during pregnancy, but instead may attributed to the mitotic activity of different cells inside these tissues, which were increased in number as well as a response to the functional status in regards to the preparation for lactation at delivery and promoting of lactation at mid lactation period. These findings may explain the increment of RNA concentrations (36). Also it has been found that activin-A restrains estrogen-induce proliferation in estrogen positive epithelial

of GH gene expression level.

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مستوى تعبير الحامض النووي الرايبوزي الرسول لجين هرمون النمو والتعبير المناعي النسجي الكيميائي للخلايا المحرضة الجسمية في الغدة النخامية لإناث جرذان الوستر الحوامل والمدرة للبن الممنعة ضد وحدة الانـهبين ألفا

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الخلاصة

تصنف الانهبينات والاكتفينات أعضاءً ضمن عائلة عوامل النمو بيتا الانتقالية المرتبطة بالفعاليات التكاثرية. تضمن العمل الحالي دراسة دور التمنيع الميسر ضد الانهبين ألفا في مستوى تعبير الحامض النووي الرايبوزي الرسول لجين هرمون النمو والتعبير المناعي النسجي الكيميائي للخلايا المحرضة الجسمية في الغدة النخامية لإناث جرذان الوستر الأباكير أثناء مرحلتي الحمل والرضاعة. تم تقسيم 48 إنثى حامل على مجموعتين (24 لكل مجموعة)، وفي اليومين الخامس والعاشر من مدة الحمل، حقنت الاولى (السيطرة) بالمحلول الفسلجي (100 مايكرولتر في البريتون)، وحقنت الثانية بالمصل المضاد للانهبين (1 مايكروغرام مذابا في 100 مايكرولتر من المحلول الفسلجي، في البريتون). وزعت كل مجموعة على ثلاث مجموعات ثانوية متساوية العدد تمت التضحية بها في اليوم السادس عشر من الحمل (مجموعة الحمل) واليوم الأول بعد الولادة (مجموعة الولادة) واليوم الحادي عشر من الرضاعة (مجموعة الرضاعة)، بعد تخديرها وتشريحها وإزالة الغدد النخامية وحفظها بدرجة -70 مئوية لغرض التقييم الجزيئي وأخرى حفظت في الفورمالين (10%) لغرض التقييم المناعي النسجي الكيميائي. أشارت النتائج الى زيادة مستوى تعبير جين هرمون النمو في نخامية مجموعة التمنيع بالمقارنة مع السيطرة وفي جميع مراحل الدراسة. أظهر الفحص المناعي التسجي الكيميائي زيادة في نسبة الخلايا الموجبة للصبغة وشدة الصبغة في نماذج المجموعة الممنعة أثناء المراحل لثلاث. وعند المقارنة بين مدد الدراسة ، أظهرت المجموعتان درجة عالية للتلوين أثناء الحمل بينما أظهرت مرحلة الرضاعة أقل درجة للتلوين. يستنتج من الدراسة أن للتمنيع الميسر ضد وحدة الانهبين ألفا في اليوم الخامس والعاشر من مدة الحمل دورا فعالا في مستوى افراز هرمون النمو من الغدة النخامية لإناث جرذان الوستر الأباكير أثناء مرحلتي الحمل والرضاعة.

الكلمات المفتاح: التمنيع الميسر، الانـهبين، الاكتفين، عوامل النمو بيتا الانتقالية، هرمون النمو، الغدة النخامية.