EFFECT OF PASSIVE IMMUNIZATION AGAINST INHIBIN-A, -BA, AND –BB SUBUNITS ON SERUM GROWTH AND DIFFERENTIATION HORMONES PROFILE IN PREGNANT AND LACTATING PRIMIPAROUS WISTER RATS

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ABSTRACT

Transforming growth factor β (TGFβ) superfamily members are closely associated with tissue remodelling events and reproductive processes. The present study aimed to determine the role of immunoneutralization of endogenous inhibin-α, βA, and βB subunits on serum growth growth and differentiation hormones profile during pregnancy, delivery, and lactation periods in primiparous female Wister rats.Eighty four pregnant primiparous female rats were assigned to 4 groups (21 per each). On 5th and 10th day of gestation, control was injected with saline (100µl, *i.p.*), Ta, Tba, and Tbb groups were injected with inhibin-α, βA, and βBantiserum (1µg in 100µl of saline, *i.p.*), respectively. Each group was allocated to 3 equal subgroups: pregnancy, delivery, and lactation subgroups were sacrificed on day 16 of gestation, 1stday after parturition, and 11th day of lactation, respectively. At the end of each subgroups period, females were anesthetized, dissected and blood samples were obtained for assessment of inhibin-A, -B, activin-A , -B, -AB, GH, and prolactin levels. Serum inhibin-A concentration in Tbb group increased during pregnancy and delivery among experimental groups. In comparison between periods, Tbb group showed significantly higher level during pregnancy and decreased during delivery and lactation, whereas Ta and Tba groups recorded no difference between periods. Inhibin-B increased in control and Tba groups during pregnancy, whereas lactation period showed higher levels in Ta, Tba, and Tbb groups compared with control. Ta, Tba, and Tbb groups recorded no siginificant differences between pregnancy and

delivery periods but they were significantly higher in lactation period.During pregnancy and delivery, Tbb group revealed higher levels of activin-A and lowest level of activin-B concentrations among groups, whereas activin-AB concentration increased in control and Ta groups. In comparison between periods, activin-A concentration was higher at delivery, whereas activin-B and –AB concentrations were higher at lactation. Serum GH, in Ta group, recorded higher level during the three periods among groups. In comparison between periods, the levels of GH and PRL in all groups showed higher levels at delivery followed by lactation and pregnancy.In conclusion, passive im munization against inhibin-α, βA, but not βB subunit, at 5^{th} and 10^{th} day of pregnancy, have ameliorating role on serum growth and differentiation hoemones profile.

INTRODUCTION

The endocrine system coordinates development of the mammary gland with reproductive development. Three categories of hormones are involved; the reproductive hormones levels (estrogen, progesterone, placental lactogen, prolactin, and oxytocin), change during reproductive development or function and act directly on the mammary gland to bring about developmental changes; metabolic hormones (GH, corticosteroids, thyroid hormone, and insulin) whose main role is to regulate metabolic responses to nutrient intake or stress, often have direct effects on the mammary gland; mammary hormones, includes GH, prolactin, and leptin (1).The ductal morphogenesis is regulated by estrogen and GH (2), whereas the proliferative phase of alveolar morphogenesis requires progesterone and prolactin (3). The mammary differentiation is called lactogenesis which was shown long ago to occur in two stages (4);lactogenesis 1 start from midpregnancy to few days prior parturition which requires progesterone and prolactin (3), while the prolactin is necessary in lactogenesis 2 in all species (5).

Activins and inhibins are members of the TGFb superfamily, a class of dimeric glycoproteins that display a wide spectrum of activities. Subsequently, they have been shown to play complex roles in neuroendocrine regulation and also modulate luteotropic hormone, growth hormone and adrenocorticotropic hormone production. In addition, they affect gonadal functions such as steroid production and regulate placental hormone synthesis (6).In βB-deficient mice, the activities of activins B and AB as well as inhibin B are eliminated (7). βB-deficient pups, develop reduced

fertility of the females(8) and a prolonged gestation time (9) may be indicative of systemic endocrine defects. Most prominently, females exhibit a lactational defect and cannot support their litters. The inactivation of the βA gene eliminates activin A and AB as well as inhibin A and causes malformation of the secondary palates and an absence of teeth and whiskers. Since the newborn mice are incapable of suckling and die within 24 hours, the effects on mammary gland development are not known. Mice deficient in both βA and βB exhibit a combination of the phenotypes seen in each of the mutants but have no additional defects (8).

Passive immunization against inhibin-α subunit causes an increase in the concentration of plasma FSH (10, 11, 12) and in the ovulation rate in the female rat (13, 14), cattle (15; 16), goats and sheep (17, 18). Also, plasma levels of estradiol-17b significantly increased in the rats treated with inhibin-antiserum (11). 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-17b, which induces the LH surge by positive feedback effect to the hypothalamus-pituitary axis (19, 20).

The present study aimed to examine the role of passive immunization against endogenous inhibin-α, βA, and βB subunits on serum growth and differentiation hoemones profile during pregnancy, delivery, and lactation periods in primiparous female Wister rats.

MATERIALS AND METHODS

Preparation of Inhibin subunitsantiserum 1%: Inhibin-α, βA, and βB 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 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. Eighty four pregnant females were randomly divided into 4 groups (21 females per each). On 5^{th} and 10^{th} days of gestation,**control (C)**females were injected with physiological saline (100µl, *i.p.*), **antiinhibin-α group (Ta)**femaleswere injected with inhibin-α antiserum

(100µl of physiological saline containing 1µg of antiserum, *i.p.*), **antiinhibin-βA group (Tba)**femaleswere injected with inhibin-βA antiserum (100µl of physiological saline containing 1µg of antiserum, *i.p.*), and **antiinhibin-βB group (Tbb)**femaleswere injected with inhibin-βB antiserum (100µl of physiological saline containing 1µg of antiserum, *i.p.*).Each group was allocated to three subgroups (7 females per each): subgroup1 (pregnancy) rats were sacraficed on the 16th day of gestation, subgroup2 (Delivery) were sacraficed on the 1st day after parturition, and subgroup3 (lactation) were sacrafied on the 11th day of lactation.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 weight, *i.p.*) (21), dissected and blood samples were obtained from abdominal vein in non haeparinized test tubes, and blood serum samples were separated for the assessment of inhibin-A, inhibin-B, activin-A , activin-B, activin-AB, GH, and prolactin concentrations in the serum.

Hormonal assays in blood serum by ELISA technique: According to the manufacturer instructions (ABO Switzerland), inhibin A and B, activinA, B, and AB, GH, and PRL, concentrations (ng/ml) have been assessed.

Statistical Analysis: Results were expressed as mean ± standard error. Comparisons were performed using one way analysis of variance (ANOVA1) 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

Serum inhibins concentrations:

During pregnancy, the higher level ($P<0.05$) has been recorded by Tbb group compared with control, Ta, and Tba groups.During delivery, the higher levels ($P<0.05$) has been registered in control and Tbb groups when compared with Ta and Tba groups. There is no siginificant difference ($P>0.05$) between groups during the lactation period. In comparison between periods, the level in control during the delivery period showed the higher level compared with pregnancy and lactation periods, which showed no difference ($P>0.05$) between each other. Whereas Ta and Tba groups recorded no difference between periods. While Tbb group results showed significantly ($P<0.05$) higher level during pregnancy and thereafter the concentration

decreased significantly (P<0.05) during delivery and continued in decrease during lactation figure (1-A).

Figure (1-B) clarify the serum concentration of inhibin-B (ng/L). During pregnancy, control females regisrered the significant (p<0.05) highest level among the experimental groups. During delivery, higher levels (p<0.05) have been recorded in control and Tba groups compared with Ta and Tbb groups. At lactation period, there is no siginificant difference (p>0.05) in serum inhibin-B concentration of Ta, Tba, and Tbb groups when compared with each other but they were significantly (p<0.05) higher than control. In comparison between periods, the concentration in control during pregnancy recorded siginificant (p<0.05) higher level compared with delivery which continued in decrement during lactation period. Ta, Tba, and Tbb groups recorded no siginificant (p>0.05) differences between pregnancy and delivery periods but they were significantly (p<0.05) higher in lactation period when compared with their coresponding concentrations in pregnancy and delivery periods.

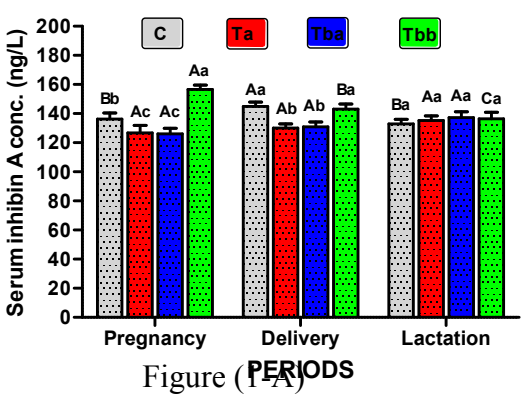


Figure (1-A)

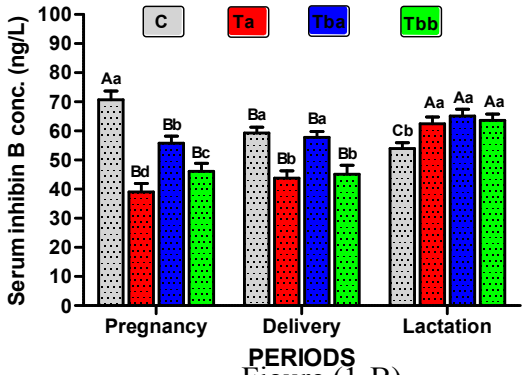


Figure (1-B)

Effect of passive immunization against inhibin-α, -βa, and –βb subunit on serum inhibin A and B, conc. (ng/L) 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.
Ta: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.
Tba: treated rats injected with inhibin-βa subunit antiserum (1µg , *ip*) on 5th and 10th day of pregnancy.
Tbb: treated rats injected with inhibin-βb subunit antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.
Serum activins concentrations:

During pregnancy, the significantly (P<0.05) higher level has been recorded by Tbb group compared with control, Ta, and Tba groups, which showed no significant (p>0.05) difference when compared with each other. Same result has been shown during delivery as Tbb group recorded the higher (p<0.05) level compared with

control, Ta and Tba. There is no siginificant difference ($p>0.05$) in serum activin-A concentrations between experimental groups during the lactation period. In comparison between periods, the concentration in all experimental groups showed same picture as the significant ($p<0.05$) higher levels have been recorded at delivery followed by pregnancy period, whereas the lowest levels recorded at lactation period (figure 1-C).

Figure (1-D) clarify the result of serum concentration of activin-B (ng/L) of the experimental groups. During pregnancy, Tbb group recorded the lowest ($p<0.05$) concentration compared with control, Ta, and Tba which showed no siginificant ($p>0.05$) difference when compared with each other. Same picture has been shown during delivery as Tbb group recorded the lowest ($p<0.05$) level compared with control, Ta and Tba. During lactation period, the results recorded no siginificant difference ($p>0.05$) in comparison between groups. In comparison between periods, the concentration in all experimental groups showed same profile as the significance ($p<0.05$) higher levels have been recorded at lactation followed by pregnancy period, whereas the lowest levels recorded at delivery period.

Figure (1-E) demonstrates the result of serum activin-AB concentration (ng/L) in the experimental groups. During pregnancy, the siginificantly ($p<0.05$) higher level has been recorded by control and Ta groups compared with Tba and Tbb groups, which showed no siginificant difference ($p>0.05$) between control and Ta groups or between Tba and Tbb groups. During delivery, the higher siginificant ($p<0.05$) level has been registered in control and Ta groups in comparison with Tba and Tbb groups, which showed no siginificant difference ($p>0.05$) between control and Ta groups or between Tba and Tbb groups.The results revealed no siginificant difference ($p>0.05$) in serum activin-AB concentration between experimental groups during the lactation period. In comparison between periods, the concentrations in all experimental groups showed same profile, as the significant ($p<0.05$) higher levels have been recorded at lactation period followed by pregnancy period, whereas the lowest levels recorded at delivery period.

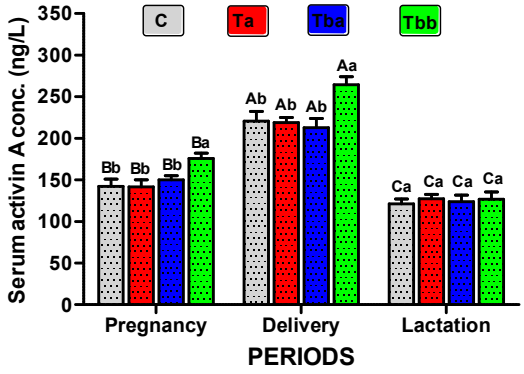
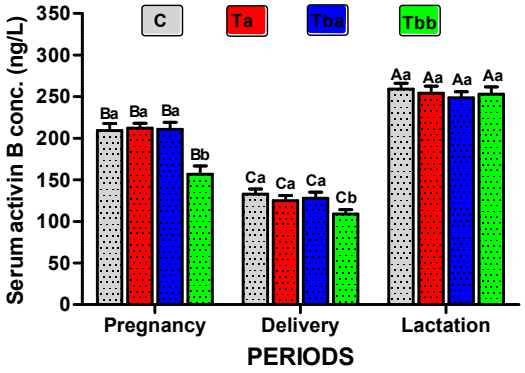


Figure (1-C)



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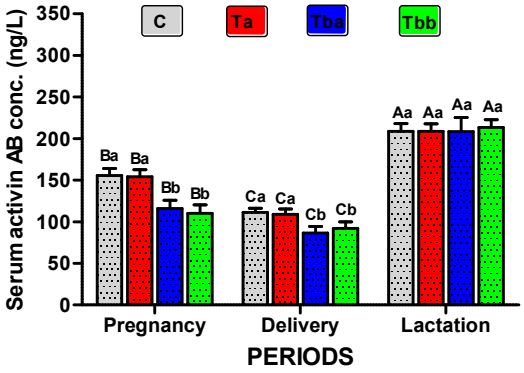

Figure (1-E)

Figures (1-C, D &E):Effect of passive immunization against inhibin-α, -βa, and –βb subunit on serum activin A , B and AB conc. (ng/L) 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.
Ta: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.
Tba: treated rats injected with inhibin-βa subunit antiserum (1µg , *ip*) on 5th and 10th day of pregnancy.
Tbb: treated rats injected with inhibin-βb subunit antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

Serumgrowth hormone concentration:

During pregnancy, the siginificant (p<0.05) higher level has been recorded by Ta group compared with control, Tba, and Tbb groups, where there is no siginificat difference (p>0.05) between Tba and Tbb which they were siginificantly (p<0.05) higher than control. During delivery, the higher siginificant (p<0.05) level has been recorded in Ta and Tba groups in comparison with control and Tbb groups, whereas there is no siginificant difference (p>0.05) niether between control and Tba groups

nor betweenTa and Tbb groups. During lactation, the siginificantly ($p<0.05$) higher level has been recorded by Ta group compared with control, Tba, and Tbb groups. In comparison between periods, the concentration in all experimental groups showed same profile as the significant ($p<0.05$) higher levels have been recorded at delivery followed by pregnancy period, whereas the lowest levels recorded at lactation period (figure 1-F).

Serumprolactin concentration:

There is no siginificant difference ($p>0.05$) between experimental groups during pregnancy period. At delivery peroid, there is no siginificant difference ($p>0.05$) in comparison between the experimental groups. Same profile has been shown during lactation period, as there is no siginificant difference ($P>0.05$) in serum prolactin concentration between control, Ta, Tba, and Tbb groups. In comparison between periods, the concentration in all experimental groups showed same profile as the significant ($p<0.05$) higher levels have been recorded at delivery period followed by lactation period, whereas the lowest ($p<0.05$) levels recorded at pregnancy period (figure 1-G).

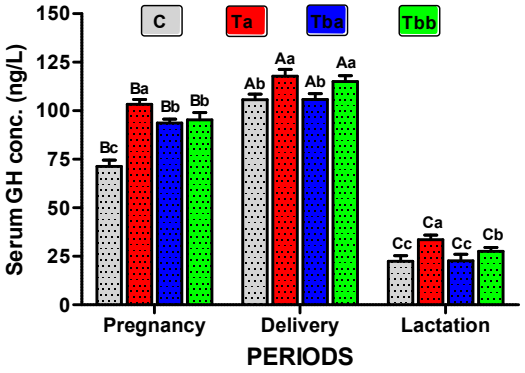

Figure (1-F)

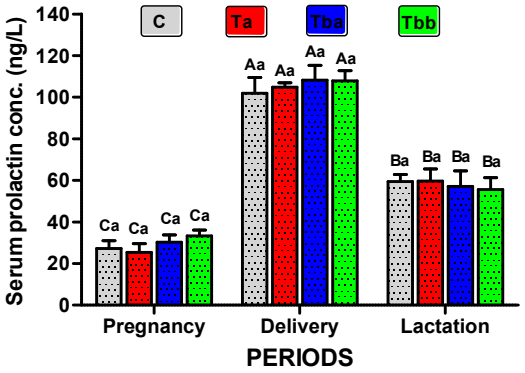

Figure (1-G)

Figure (1-F & G): Effect of passive immunization against inhibin-α, -βa, and –βb subunit on serum GH, and PRL conc. (ng/L) 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.
Ta: pregnant rats injected with inhibin-α antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.
Tba: treated rats injected with inhibin-βa subunit antiserum (1µg , *ip*) on 5th and 10th day of pregnancy.
Tbb: treated rats injected with inhibin-βb subunit antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.

DISCUSSION

During pregnancy, the level of inhibin-A decreased in Ta group compared with control due to the immunoneutralization against inhibin-α subunit, as it cosidered the unique constituent of inhibins (22), therefor the secretion of the two types of inhibins (A and B) could be decreased. Also, the level of inhibin-A decreased in Tba group compared with control due to the immunoneutralization against inhibin-βA subunit, as it considered as one of the two main constituents of inhibin-A (22),whereas the level of inhibin-A increased in Tbb group because the immunoneutralization was against inhibin-βB subunit, while inhibin-A composed of α and βA subunit.These changes continued at delivery, but during lactation it returned to the insignificant levels because of the diminish of immunoneutralization effect, and inhibin-A could secreted again. It has been mentioned that inhibin-A peaking was in the early gestation, where pituitary Gn secretion decreased during this period, also the other studies revealed that inhibin regulates FSH secretion by reducing the amount of activin available at the binding site and also by reducing activin binding with activin type II receptors (23).The result recorded at the delivery period could related to the drop showed in activin-A and FSH levels due to immunization against inhibin βA subunits and expulsion of placenta, the main source of activin-A (24). During the lactation period no difference in the inhibin-A between groups when has been shown, the absent of inhibin-A action during lactation period attributed to the the post-partum immunoreactive inhibin and inhibin-A fall markedly and a subsequent rise in FSH is associated with an increase in inhibin-B level (25,26).

The decreased levels of inhibin-B during pregnancy in immunized groups can be explained that passive immunization against inhibin-α subunit (Ta group) and inhibin-βB subunit (Tbb group) could decrease the concentration of inhibin-B, where inhibin-B composed of inhibin-α and βB subunits (22). On the other hand, the decrease of inhibin-B in Tba group may attributed to the rise of FSH secretion, as it has been mentioned that inhibin-B decreased coincident with the first detection of FSH (25,27).These changes remained at delivery except the difference between control and Ta group, which may attributed to the high activity of activins, as a proliferative factors, during mammogenesis at and after delivery (28). At mid lactation, inhibin-B recorded significant incresed in all immunized groups. This increment may related to the increase of gonadotropin secretion (mainly FSH) due to the significant decrease in prolactin as mentioned in the present study, it has been mentioned that prolactin level regulats hypothalamic GnRH secretion and pituitary gonadotropin secretion (29). On

the other hand, it has been mentioned that suckling result in an increase release of β-endorphin, which result in supression of gonadotropin level via inhibition GnRH secretion (30).The present changes accompanied by the increase of serum prolactin and GH levels.

The result of immunoneutralization against inhibin-βA subunit caused significant increase of activin-A in Tbb group during pregnancy and delivery, but it returned to the non significant level at mid lactation, whereas immunoneutralization against inhibin-α and inhibin-βA didn't change the level of inhibin-A at all stages of the study. The decrease of inhibin-A in Ta and Tba groups compared with Tbb group could attributed to the neutralization of βA subunit which decreased the biosynthesis of inhibin-A, activin-A and activin-AB, where this subunit is the main constituent of these factors, whereas the immunization against βB not related to activin-A but instead related to inhibin-B, activin-B and -AB, as they consists of α- βB, βB- βB, and βA- βB, respectively (6). During mid lactation the level of activin-A recorded no significant differece between groups, because the effect of immunization diminished during this period. On the other hand, in comparison between periods, all groups showed significant increase at delivery compared with pregnancy period, and tend to significant decrease at mid lactation. These flactuation could be attributed to the expulsion of placenta after vaginal delivery, the site of activin-A secretion (31). Activin-A and follistatin present in milk, begining at the first day of lactation and continuted through the lactation (32). It has been found that activin-A involves, at delivery, in the mechanisim of labor by stimulation of

the release of prostaglandins and oxytocin (33). Also activin-A inconsert with estradiol in supression of FSH during pregnancy. Therefore it appearthat activin-A has important role during mid to late pregnancy and parturition as well as preparation of mammary gland for lactation.The high level of activin-A in Tbb group, at pregnancy and delivery accompanied by low level of activin-B. These changes could attributed to the depletion of inhibin-βB subunit which considered as the main constituent of activin-B (22).

On the other hand, the present findings mentioned to significant decrease of activin-B at delivery, whereas its concentrations was higher significantly during pregnancy and mid lactation. Activin-B is required for branching and alveologenesis (34), also it has been mentioned that activin-B subunits expressed greatest from mid to late lactation but decreased during involution (28). Furtheremore, the

downregulation of activin-B accompanied by high level of estradiol which increase during the delivery (35,36).

Immunization of female rats against inhibin-βA (Tba group) and against inhibin-βB (Tbb group) reduced the concentration of activin-AB in these groups at both pregnancy and delivery periods but not at mid lactation. The decrease at pregnancy and delivery, actually due to the depletion of βA subunit in Tba group and βB subunit in Tbb group, since activin-AB is composed from βA- βB subunits. During pregnancy, the levels of activin-AB, in all groups, were higher than delivery period which could attributed to the expulsion of placenta after vaginal delivery, as placenta is the main source of activin-AB secretion (31). Whereas the highest levels of activin-AB were recorded at lactation in all groups, since mammary gland is the source of activin-AB during lactation. It has been reported that activin-A, activin-B, and activin-AB proteins along with their receptors have been expressed in the mouse mammary gland during lactation (37).

Growth hormone:

During pregnancy, the high level of GH, recorded in immunized groups could be attributed to the decrease in inhibins, as it has been reported by Thanoon (11) that passive immunization against inhibin-α subunit increased expression levels of hypothalamic GHRH and pituitary GH genes. The higher concentration of GH in Ta group may be attributed to the role of inhibin α antiserum which neutralize both types of inhibins (A and B), whereas immunization against βA and βB subunits neutralized either inhibin-A or inhibin-B, respectively.At delivery, all groups recorded significant elevation compared with their corresponding levels at pregnancy, with the remaining of differences in Ta and Tbb groups. It seems that Inhibin-A protein is more important in regulating GH secretion than inhibin-B. Also the immunization against βB subunit could decrease the levels of activin-B and activin-AB but increase the level of activin-A. Therefore, activin-A decreases the secretion of GH, as it has been registered that activin-A being able to inhibit basal GH secretion from pituitary gland (38-40). During this period, the elevation of GH may be attributed to the increase in serum estradiol secretion at the end of gestation (41). At mid lactation, the level of GH decreased sharply in all groups, which accompanied by significant increase in inhibin-B, activin-A, and activin-AB (11).

Ta groups, at all experimental period, revealed higher levels of GH among experimental groups which may be attributed to the increase of activins and estradiol

levels after immunoneutralization, 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. Upon pregnancy, the combined actions of progesterone and prolactin generate alveoli, which secrete milk during lactation. Lack of demand for milk at weaning initiates the process of involution whereby the gland is remodeled back to its prepregnancy state. These processes require numerous signaling pathways that have distinct 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 (42).

Prolactin

Prolactin concentrations showed no significant difference between experimental groups at all periods, while comparison between experimental periods revealed significant elevation of prolactin concentration at delivery compared with other period and then tend to decrease at mid lactation, but its level still significantly higher than that during pregnancy period. The elevation at delivery could be attributed to sharp increase of estradiol and the high level of progesteron, as it has been mentioned that estradiol promotes prolactin secretion from pituitary gland by inhibiting dopamine (43). Activins (namely activin-B and activin-AB) act as negative regulator of prolactin expression and secretion in the pituitary gland (44).Our result was in agreement with the studies which illustrate that, in the rats, the fall in the circulating progesterone is followed by an increase in serum prolactin (45), whereas the lowest level during pregnancy is due to higher level of activins which act as a negative regulator of prolactin expression and secretion in pituitary culture and cell lines (44). Also, as the ovarian hormone, estrogen, is critical regulator of pubertal mammary development and is responsible for the tremendous surge in growth occurring during this period that generates a functional mammary gland. For a long time, it was unclear whether hormones such as estrogen had direct effects on mammary gland development or whether, instead, they functioned indirectly to stimulate the release of hormones such as PRL from the pituitary (46).

تأثير التمنيع الميسر ضد وحدات الانهبين ألفا وبيتا أي وبيتا بي على مستوى هرمونات النمو والتخصص في أباكير جرذان الوستر الحوامل والمدرة للبن

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الخلاصة

لأعضاء عائلة عوامل نمو بيتا الانتقالية TGF-β علاقة وثيقة في توجيه الأحداث الخاصة بتجديد الانسجة والتكاثر. هدفت الدراسة الحالية لاختبار دور التمنيع ضد وحدات الانهبين (ألفا و بيتا أي وبيتا بي) في اليوم الخامس و العاشر من مدة الحمل في مستوى الانهبين أي وبي والاكتفين أي وبي وأي بي وهرموني النمو والبرولاكتين في مصل الدم.تم تقسيم ٨٤ من إناث الجرذان الحوامل على أربع مجموعات (٢١ لكل مجموعة). في اليوم الخامس والعاشر من مدة الحمل، تم حقن إناث السيطرة بالمحلول الفسلجي (١٠٠ مايكرولتر في البريتون)، وحقنت مجموعات التمنيع (Ta و Tba و Tbb) بالمصل المضاد للانهبين أي وبيتا أي وبيتا بي (١ مايكروغرام مذابة في ١٠٠ مايكرولتر من المحلول الفسلجي، في البريتون)، على التوالي. قسمت كل مجموعة الى ٣ مجاميع ثانوية (٧ إناث لكل منها)، تمت التضحية بها في اليوم ١٦ من الحمل وفي اليوم الأول بعد الولادة واليوم ١١ من مدة در اللبن، وأخذت منها عينات دم لقياس مستوى الانهبين أي وبي والاكتفين أي وبي وأي بي وهرموني النمو والبرولاكتين في المصل.سجلت مجموعة Tbb أعلى مستوى للانهبين أي في المصل أثناء مدة الحمل، وأثناء الولادة سجل أعلى مستوى في مجموعتي السيطرة و Tbb. ، سجلت السيطرة أعلى مستوى خلال مدة الحمل، وسجلت مجموعة Tbb أعلى مستوى خلال الحمل. أظهرت مستويات الانهبين بي في مجموعة السيطرة أعلى المستويات خلال مدة الحمل بينما كان أعلى مستوى له أثناء الولادة في مجموعتي السيطرة و Tba في حين كانت المستويات في المجاميع الممنعة متقاربة فيما بينها خلال مدة در اللبن إلا أنها أعلى معنويا من السيطرة. وعند المقارنة بين مدد الدراسة، وجد في السيطرة أعلى مستوى له أثناء الحمل وعدم وجود فرق بين مدتي الحمل والولادة للمجموعات الممنعة الثلاث إلا أنها أعلى من مدة در اللبن. سجلت مستويات الاكتفين أي خلال مرحلتي الحمل والولادة في مجموعة Tbb إرتفاعاً من بين مجموعات الدراسة بينما لم تظهر فروقات بين المجاميع أثناء مدة در اللبن. وعند المقارنة بين مدد الدراسة، أظهرت جميع المجاميع ارتفاعاً أثناء الولادة. أما الاكتفين بي فقد سجل أقل المستويات في مرحلتي الحمل والولادة في مجموعةTbb بينما لم يسجل فرقاً خلال مدة در اللبن. وعند المقارنة بين المدد سجلت كل المجاميع أعلى المستويات خلال مدة در اللبن. كانت مستويات الاكتفين أي بي في مجموعتي السيطرة و Ta هي الأعلى في مرحلتي الحمل والولادة. وعند المقارنة بين مدد الدراسة، أظهرت جميع المجاميع أعلى مستوى لها أثناء مدة در اللبن. سجلت معدلات هرمون النمو في مجموعة Ta أعلى المستويات من بين مجاميع الدراسة خلال الحمل، وأثناء الولادة سجلت أعلى المستويات في مجموعتي Ta و Tba بينما أشارت مرحلة در اللبن إلى أن أعلى المستويات كانت في مجموعة Ta أيضاً. وعند المقارنة بين مدد الدراسة، كانت المستويات في جميع المجاميع أعلى أثناء الولادة. ، ولكن المقارنة بين مدد الدراسة

أشارت الى أن المستويات في كل المجموعات كانت الأعلى أثناء الولادة. نستنتج بأن التمنيع ضد وحدات الانهيبين (الفا وبيتا أي) في اليوم الخامس والعاشر من الحمل له دور بتحسين عمل وتأثيرات هورمونات النمو والتخصص في مصل الدم والمؤثرة على نمو وتطور الغدد اللبنية.

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