Effect of Inhibin-α, -βA, and βB Immunoneutralization at Pregnancy on Mammary PRL-r Gene and Prolactin Expression Level in Pregnant, Delivered, and Lactating Rats

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Abstract

Prolactin is the main lactogenic hormone of pituitary source. To investigate the role of passive immunization against inhibin-α, βa, or βb subunits at pregnancy on mRNA expression levels of prolactin receptors (PRL-r) and immunehistochemical expression of prolactin (PRL)in the mammary glands of pregnancy, delivered and lactating female rats. Ninety six pregnant females were assigned into four groups (24 each).On 5^{th} and 10^{th} day of pregnancy, first group was injected with physiological saline (100µl, *i.p.*) and served as control(C), and other groups were *i.p.* injected with 1µg in 100µl of saline of inhibin-α, -βaand -βb antisera, respectively (Ta, Tba, and Tbb). Eight females from each group were sacrificed on the 16^{th} day of gestation, the 1^{st} day after parturition, and the 11^{th} day of lactation. Mammary gland samples from each female were obtained for evaluation of the expression levels of prolactin and prolactin receptors gene. During pregnancy, PRL expression levels of in Tbb group increased significantly among experimental groups, whereas delivery and lactation periods recorded higher expression in Ta and Tbb than Tba.PRL-r gene expression levels, during pregnancy, registered significant elevation in Tbb group, whereas at delivery and lactation, significant elevation has been shown in Ta group. It can be concluded that activins (mainly activin-A)have potency in mammary growth and development early at mid pregnancy.

KEYWORDS: mammary gland, PRL, PRL-r, passive immunization.

Introduction

Inhibin, a glycoprotein hormone delivered by the granulosa cells in the ovarian follicles smothers the synthesis of follicle stimulating hormone (FSH) by pituitary gonadotropins, through negative feedback action. It is likewise required in the direction of folliculogenesis through autocrine and/or paracrine control (Findlay, 1993). Inhibins and activins are members of the TGF-β family of growth factors. Inhibin is composed of α-subunit and either βA (inhibin-A) or βB (inhibin-B) subunit, whereas activin is composed of two β subunits, to form activin-A (βA and βA), activin-B (βB and βB), and activin-AB (βA and βB). Activins serve as inhibin antagonist by competition to interacting on the same receptors, and accordingly removal of the inhibitory stimulatory effects of inhibins allows activins to insert their augmented action GnRH inside gonadotrophs to increase production of FSH (Bilezikjian et al., 2004; 2006).

Passive immunization against inhibin brought FSH discharge up in the period of the estrous cycle with high estradiol emission (Kaneko et al., 1995) or with high progesterone discharge (Kaneko et al., 1993). Manifold ovulations were supported viably

by immunoneutralization of endogenous inhibin in various species, for example, mice (Wang et al., 2001), rats (Al-Sa'aidi and Samir, 2010), hamsters (Kishi et al., 1996), dairy animals (Akagi et al., 1997; Takedomi et al., 1997), and female horses (Nambo et al., 1998).

The growth and development of the mammary gland requires each of the reproductive hormones (estrogen, progesterone, placental lactogen, and PRL), metabolic hormones (GH, corticosteroids, thyroid hormone, and insulin) as well as the local mammary hormones (local GH, PRL, and leptin (Neville *et al.*, 2002). The morphogenesis of mammary ducts is managed by estrogen and GH (Hovey et al., 2002), while the alveoli requires progesterone and prolactin (Brisken et al., 2002). Mammary lactogenesis which was indicated long prior to happen in two phases (Hartmann et al., 1973).Lactogenesis 1 begin from mid-pregnancy to few days earlier parturition which requires progesterone and prolactin (Brisken et al., 2002), while the prolactin is vital in lactogenesis 2 in all species (Schams, 1972). The advancement of alveolar morphogenesis phases happen amid pregnancy which begin by proliferative stage (ductal and alveolar multiplication) in early pregnancy, secretory initiation in mid pregnancy and some portion of secretory enactment stage at around parturition and continue amid lactation (Anderson et al., 2007). In rats, a fast increment in the number and size of alveolar happen after 10 days of pregnancy (lactogenesis I) bringing about the improvement of completely distinguished secretory lobules (Pitelka et al., 1973), where the mammary epithelial cells grows secretary cells (lactocytes) with ability to produce milk components, for example, lactose, casein, α– lactalbumin, unsaturated fatty acids (Anderson et al., 2007). Amid this stage, differentiated alveoli appeared (Brisken and Rajarom, 2006; Richert et al., 2000), during which prolactin activities are mostly assumed by PRL-r and GH-r (Neville et al., 2002).

Enhanced reproductive action of mammals requires elevation of FSH secretion from the adenohypophesis to empower ovarian small follicles for development. In this circumstance, it is required to have surge of FSH secretion to bolster follicular development to the point where LH receptors start to be dynamic inside granulosa cells of the full grown follicle. The key positive controllers of FSH emission are hypothalamic GnRH, pituitary activin, as well as pituitary TGF-β, while the key negative controllers of FSH discharge are gonadal inhibins and additionally pituitary and gonadal follistatin (Rozell and Okrainetz, 2009). The current study aims to investigate the role of passive immunoneutralization against the three subunits of inhibins and activins (inhibin-α, βA, and βB) on pregnant female rats mammary prolactin distribution and PRL-r upregulation at the third trimester of gestation, at delivery, and mid lactation period.

Materials and Methods

Preparation of Inhibin subunits antiserum 1%: According to the manufacturer instructions, inhibin-α, βA, and βB antibodies have been prepared in a concentration of (1µg/100µl) (ABO, Switzerland).

Experimental Animals: This study was approved for conducting animal study according to the ethical guidelines and policies of AL-Qadisiya University, Iraq. Virgin female Wistar rats (weighted 136±5.1g. and aged 80 days), were housed under laboratory conditions (12L:12D cycles at 20-22 C°) and fed on standard food (19% protein ratio and 3000 k.cal.) and drinking water *ad libitum*.

Experimental design: Ninety six females were mated with experienced males (1 male with 2 females). The appearance of vaginal plug was an indication of beginning

pregnancy. The females were randomly assigned to 4 groups (24 each). On 5th and 10th days of pregnancy, first group females were injected with physiological saline (100µl, *i.p.*), and served as control (C group), whereas other 3 groups females were injected with inhibin-α antiserum (1µg %, *i.p.*) (Ta group), inhibin-βA antiserum (1µg %, *i.p.*) (Tba group), and inhibin-βB antiserum (1µg %, *i.p.*) (Tbb group). Each group was allocated to three subgroups (8 females each): subgroup1 (pregnancy) females were sacrificed on the 16th day of pregnancy, subgroup2 (Delivery) females were sacrificed on the 1st day after parturition and in subgroup3 (lactation) litters number was modulated as 9 per each dam (Tucker, 1987) and females were sacrificed on the 11th day of lactation. At the end of each subgroup period, each female was anesthetized (by injection of 0.3 ml ketamine + 0.1 ml xylazine/kg body weight, *i.p.*) (Sharpe and LaRegina, 1998), dissected and two samples from mammary glands were obtained. First sample was quickly kept at -80 ºC for theevaluation of mRNA expression levels of GAPDH and PRL receptor (PRL-r) gene using qRT-PCR based on Syber Green dye, and second sample was kept in formalin 10% and processed in paraffin blocks for immunohistochemical (IHC) staining technique to examine PRL staining density and score.

Quantitative Reverse Transcriptase Real-Time PCR: According to the method mentioned in Wang and Hardy (2004), qRT-PCR technique was used for quantification of *PRL-r* gene expression levels relative to Housekeeping gene in the mammary gland. Data analysis of qRT-PCR assay included primer efficiency estimation and relative quantification of *PRL-r*gene expression levels normalized by housekeeping gene expression (*GAPDH*). Threshold cycle numbers (Ct) were calculated from amplification plot of RT-PCR detection system, during exponential phase of fluorescent signals of SYBR®green primer the gene that react with cDNA of rat mammary gland mRNA, where, the amount of DNA copy numbers(PCR product) in master mix reaction is doubles in each PCR cycle. First prepared series dilution of mammary glands cDNA of control females were used with the primer of *PRL-r* gene to form the amplification plot, and from this amplification plot, threshold cycle (Ct) was used to calculate a linear regression based on the data points, and inferring the efficiency of each primer from the slope of the line. The relative quantification of target genes expression in mammary gland has been calculated using the $2^{\wedge-\Delta\Delta Ct}$livak and Schmittgen method. The expression of control gene was used as calibrator in both target and reference gene (*GAPDH*). At first, the threshold cycle number of target gene was normalized to that of reference gene in treatment groups and calibrator. Second, the ΔCt of treatment groups 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).

Immunohistochemistry-Paraffin protocol: According to Luna (1968), histological sections were prepared from mammary glands. According to the manufacture instructions (Abcam, UK; **www.abcam.com/technical)**, immunohistochemistry has been performed for demonstrating the presence and score of PRL in the mammary gland sections.

Statistical analysis: Results were expressed as mean ± standard deviation. Comparisons between groups and periods values were performed using one way analysis of variance (ANOVA1) and newman- keuls. Differences were considered to be significant at the level of $P<0.05$. Statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA).

Results:

Relative quantification of mammary *PRL-r* gene expression: Figure (1) illustrates the fold changes of *PRL-r* gene in the mammary gland tissues obtained from treated female rats during pregnancy, delivery, and lactation periods. During pregnancy, the expression levels of *PRL-r* gene increased significantly ($p<0.05$) in Tbb group compared with Ta and Tba groups, which showed significant difference when compared with each other. At delivery and lactation, significant ($p<0.05$) elevation of *PRL-r* gene expression levels have been shown in Ta and Tbb groups compared with Tba group.

Immunohistochemical staining of mammary PRL: Prolactin staining density that has been detected in mammary tissue of Tbb group female rats, during pregnancy was significantly higher among experimental groups. At delivery and lactation, mammary tissues obtained from Ta and Tbb females showed higher density than control and Tba females. In comparison between periods of the experiment, in all experimental groups, higher score of staining has been shown at the 1st day after parturition, whereas the lowest score recorded at the 16th day of pregnancy (figure 2).

Discussion

Milk yield for the most part thought to be restricted by mammary size (number of secretory cells) and cellular activity. Significant exploration exertion has been coordinated toward upgrading mammary capacity and increasing set up lactation. Moreover, the likelihood exists that induction of mammary growth and improvement (either at adolescence or amid pregnancy) could increment lactational execution. Due to the exponential status of mammary gland improvement shown in many species, moderately little changes in the development rate of the mammary gland amid pregnancy conceivably can bring about expansive changes in extreme mammary size and lactational activity (Sheffield and Anderson, 1985).

From our observations, immunization against inhibin-βB caused elevation of PRL and PRL-r in mammary tissue during pregnancy, therefore it can be suggested that inhibin-A and/or activin-A have an augmented role in the development or in the preparation of mammary gland for lactation, since other members (inhibin-B, activin-B, and activin-AB) have been neutralized due to injection of inhibin-α and –βA antisera. These effects continued even at delivery and mid-lactation periods. In βB-lacking mice, it has been shown that gestational period delayed, which could because of endocrine disterbances, and most unmistakably, females display a lactational desert and can't bolster their litters (Vassalli et al., 1994). It has been found that injection of inhibin-α subunit and non-steroid follicular fluid antisera causes significant delay in gestational period and progress in mammary gland ductal elongation and alveolar morphogenesis (Al-Sa'aidi and Baqir, 2014) suggesting that activins, as an antagonistic to inhibins, play a positive role in mammary gland growth and development through increasing the ductal trees and number of secretory epithelial cells that lined the alveoli.

Vale *et al.*, (1994) mentioned that activins regulate placental hormone synthesis as well as its gonadal stimulation of steroid biosynthesis. On the other hand passive immunization against inhibin-α subunit causes an increase in the concentration of plasma FSH and estradiol (Thanoon, 2013, Abdulla, 2013). Al-Sa,aidi et al. (2014) mentioned that the elevation of prolactin concentration at delivery could attributed to sharp increase of estradiol and the low level of progesterone, as it has been reported that estradiol promotes prolactin secretion from pituitary gland by inhibiting dopamine (Norman and

Litwach, 1997). The present 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 (Bussmann and Deis, 1979), whereas the lowest level during pregnancy is due to higher level of activin-B and AB which act a negative regulator of prolactin expression and secretion in pituitary culture and cell lines (Murata and Ying, 1991).

In rats, it has been mentioned that the surge of prolactin markedly eliminated at mid pregnancy when placental lactogen (PL) replaces as the major lactotrophic hormone, where a further surge of prolactin reinitiated a day before delivery (Andrews et al., 2001). In our study, it has been found high reactivity for PRL at mid pregnancy, which may attributed to the cross reaction between PRL and placental lactogen.

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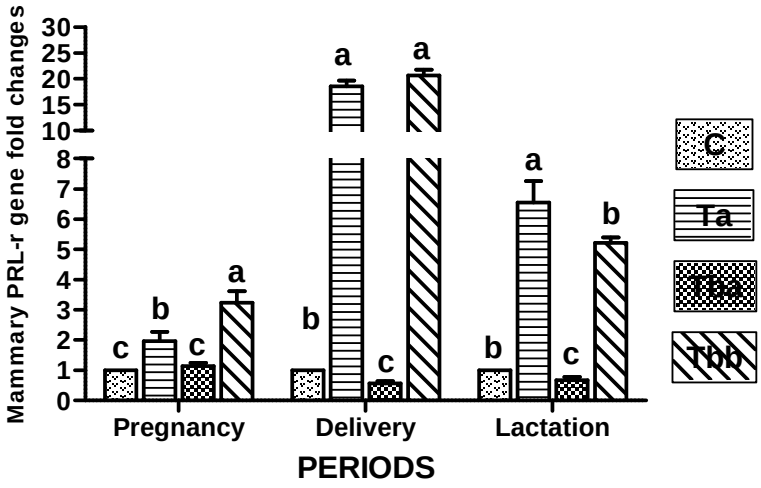


Figure (1): Mammary *PRL*-r gene expression levels (fold changes) in female rats passively immunized against inhibin-α, -βa, and –βb serotypes, during pregnancy, delivery, and lactation.

Values represents mean ± standard deviation.
Different small letters represents significances ($p<0.05$) in comparison between groups.
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.

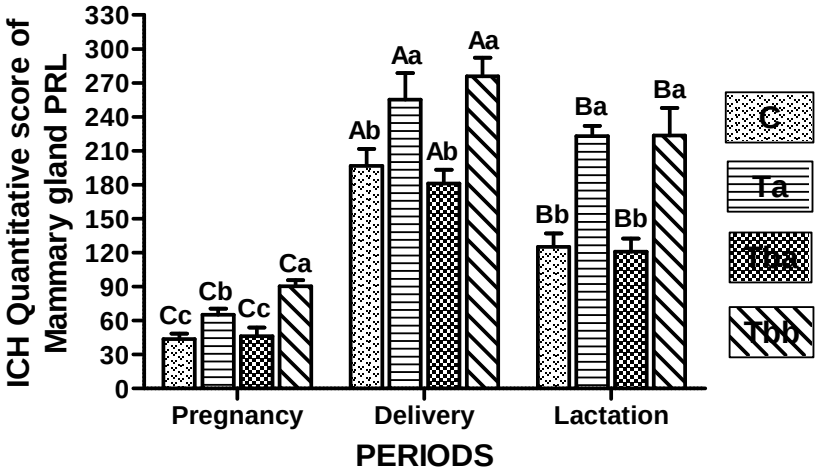


Figure (2): Quantitative IHC score of mammary PRL in female rats passively immunized against inhibin-α, -βa, and –βb serotypes, during pregnancy, delivery, and lactation.

Values represents mean ± standard deviation.

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

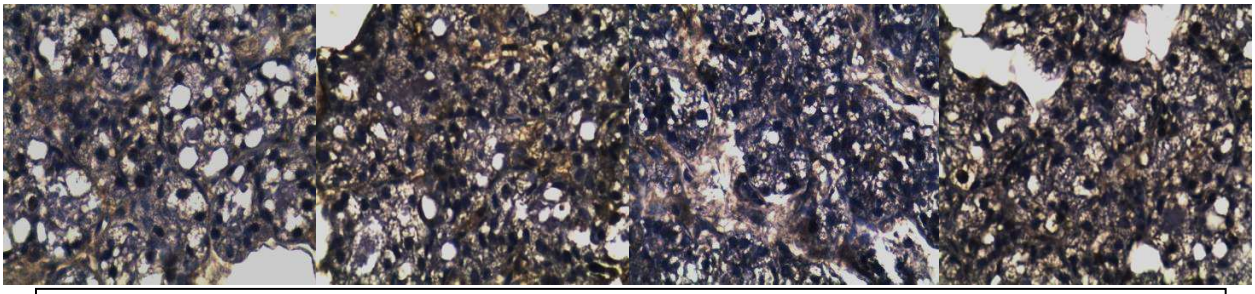
Different capital letters represents significances ($p<0.05$) in comparison between periods.

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.

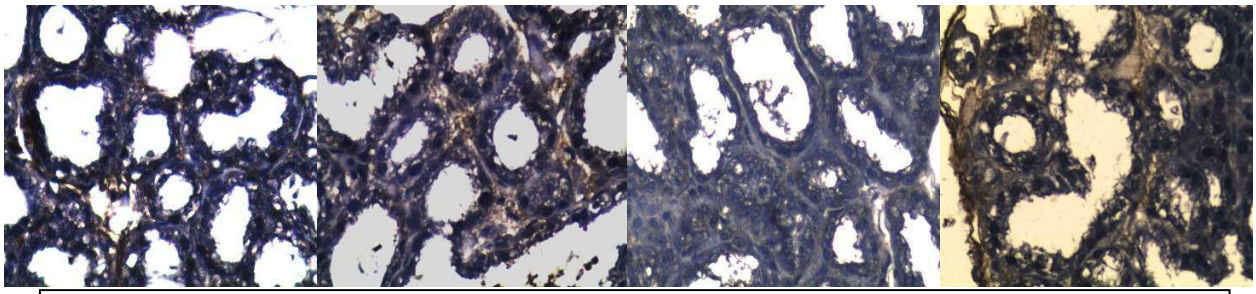
Tba: treated rats injected with inhibin-βa subunit antiserum (1µg ,*ip*) on 5th and 10th day of pregnancy.

Tbb: treated rats injected with inhibin-βb subunit antiserum (1µg, *ip*) on 5th and 10th day of pregnancy.



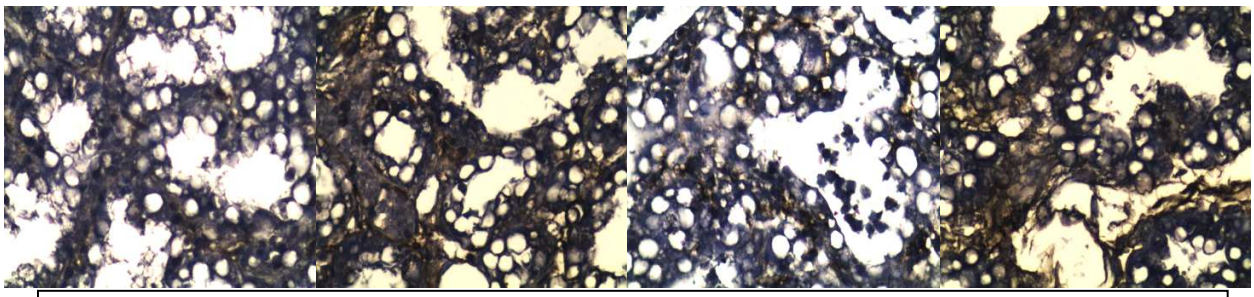
C TaTbaTbb

(A): at the 16th day of pregnancy



C TaTbaTbb

(B): at the 1st day after delivery



C TaTbaTbb

(C): at the 11th day of lactation

Figure (3): Mammary gland from control (C), inhibin-α (Ta), inhibin-βA (Tba), and inhibin βB (Tbb) antisera injected female rats, at the 16th day of pregnancy (A), the 1st day after parturition (B), and the 11th day of lactation (C), and reveals the density of actively staining PRL with immunohistochemistry. Immunohistochemistry, 500X.