

## Establishment and comparison of two methods to produce a rat model of mammary gland hyperplasia with hyperprolactinemia

Caikui Luo<sup>1</sup>, Yanming Wang<sup>2</sup>, JiliZou<sup>1</sup>, Jinhu Wu<sup>1</sup>,  
Junhua Meng<sup>1</sup>, Hanmin Zhou<sup>1</sup>, Yonggang Chen<sup>1\*</sup>

<sup>1</sup>The Third Hospital of Wu-han, Wuhan, hubei, China; <sup>2</sup>Medical school of Shihezi University, Shihezi, China

This study aimed to establish and compare models of mammary gland hyperplasia (MGH) with hyperprolactinemia (HPRL) using two different methods. The models provide information on the relationship between mammary gland hyperplasia and associated hormones. Model A was constructed using intramuscular injections of estradiol benzoate injection (EBI), followed by progesterone (P), and then metoclopramide dihydrochloride (MDI). Model B was designed by administering MDI, follow by EBI, and then P intramuscularly. Model B showed higher MGH progression compared with model A. Notably, increase in estradiol (E2) was negatively correlated with prolactin (PRL) secretion. However, PRL levels in model B were significantly higher compared with the levels in model A. Estrogen (ER), prolactin receptor (PRLR), and progesterone receptor (PR) mRNA and protein expression levels in model B rats were positively correlated with changes in the corresponding hormone levels. However, E2, P, and PRL levels in model A showed no direct relationship with levels of the mRNAs of related hormones and protein expression levels. Our results suggest that model B is an appropriate model of MGH with HPRL that can be used to perform further studies about the interactions of the E2, P, and PRL hormones in this disorder.

**Keywords:** Estradiol. Progesterone. Prolactin. Hyperplasia of mammary. Hyperprolactinemia.

### Abbreviations:

EBI, estradiol benzoate injection; PI, progesterone injection; MDI, metoclopramide dihydrochloride injection; E2, estradiol; P, progesterone; PRL, prolactin; HPRL, hyperprolactinemia; HMG, hyperplasia of mammary gland; estrogen receptor, ER; progesterone receptor, PR; prolactin receptor, PRLR.

### INTRODUCTION

Studies report that both mammary gland hyperplasia (MGH) and hyperprolactinemia (HPRL) are associated with abnormal secretion of pituitary-gonadal hormones (Riecher-Rossler 2017; Frank, Brown, Clegg, 2014). In our preliminary search, we explored pharmacodynamics

of medications used for HMG and HPRL treatment. Rat models with high HRPL levels showed high risk of MGH and significantly lower estradiol (E2) levels compared with the control group (Chen *et al.*, 2017). In contrast, high E2 levels were observed in MGH rat model compared the levels in the control group (Sun *et al.*, 2017). Further, significantly higher prolactin (PRL) levels were reported in a different MGH rat model compared with levels in the control group (Wang *et al.*, 2013a). Further, PRL levels were positively with HPRL levels, which were consistent with a previous study (Akbas *et al.*, 2013). These studies report association of pathologic changes in rat mammary glands with HPRL and E2 levels. Therefore, we speculated that MGH and HPRL disorders may develop concomitantly and influence interactions of E2, progesterone (P), and PRL hormones.

Animal models on MGH or HPRL, but not both, are used for studying the effect of interacting hormones. In

\*Correspondence: Y. Chen. The Third Hospital of Wu-han. Wuhan, hubei, 430060, China. Phone: 15102781829. E-mail: cyg508@163.com

addition, rat model studies using either MGH or HPRL separately are cumbersome and time-consuming to perform, as they require use of a large number of animals (Chen *et al.*, 2015; Raghuthaman, Venkateswaran, Krishnadas, 2015; Jia *et al.*, 2017). Therefore, there is need to develop animal model with the clinical symptoms of the two disorders at the same time in the same rat.

## MATERIAL AND METHODS

### Material

#### Chemicals

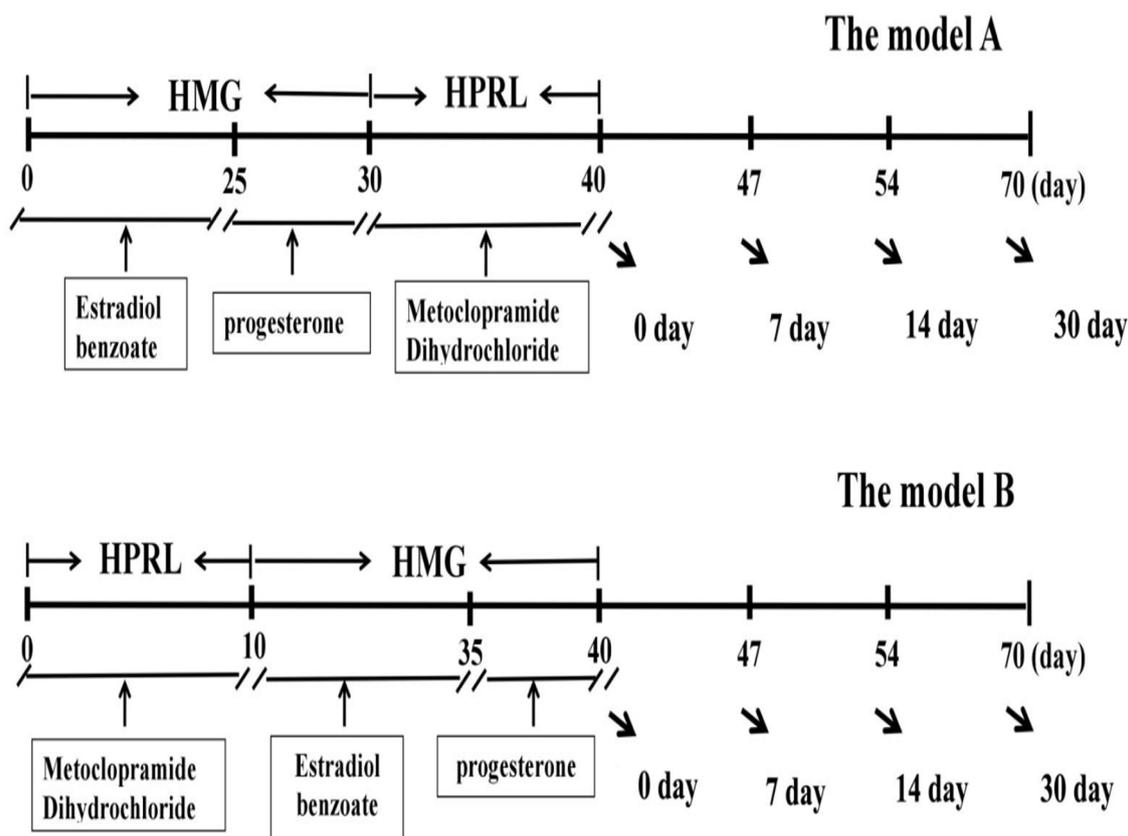
Estrogen benzoate injection was purchased from Ningbo Second Hormone Factory (Ningbo, China). Progesterone injection was purchased from Shanghai General Pharmaceutical Co., Ltd (Shanghai, China). Metoclopramide dihydrochloride injection was purchased from Absin Bioscience Inc (Shanghai China). The concentrations of PRL, E2, and P in rat sera were determined using enzyme-linked immunosorbent assay (ELISA) (Calbiotech, USA). All other reagents and solvents were of analytical grade.

#### Animals and treatments

Ninety virgin female Wistar rats weighing 200-250g (6-8 weeks old) were obtained from the Center for

Disease Control and Prevention of Hubei. Experimental protocols (NO.42000600008183) followed standards and policies of the Third Hospital of Wu-han animal ethics committee. The rats were housed in standard animal cages with temperature between 22°C-25°C and relative humidity of 50%-60%. A 12 h light-dark cycle was maintained the rodents were fed with chow and water *ad libitum*. The rats were randomly divided into three groups: model group A (hyperplasia + HPRL) (n=40), model group B (HPRL + hyperplasia) (n=40) and the normal group C (n=10).

Two methods were used to establish models of mammary gland hyperplasia with hyperprolactinemia (Figure 1). Model group A: In this group, rats were intramuscularly administered with estradiol benzoate (50mg/kg) for 25 days. Further, rats in this group were intramuscularly administered with progesterone (50mg/kg) for 5 days. Finally, 75mg/kg metoclopramide dihydrochloride injection was administered subcutaneously on the back of each rat in this group daily for 10 days. Model group B: Metoclopramide dihydrochloride injection (75mg/kg) was administered to each rat daily for 10 days. Subsequently, estradiol benzoate injection (50mg/kg) was administered daily for 25 days, followed by progesterone injection (50mg/kg) administration daily for 5 days. Normal group C: Rats in this group were intramuscularly given saline (0.1 mL) for 40 consecutive days.



**FIGURE 1** - Behavioral experimental schedule for model A and B.

**Methods**

*Hormone assay and Histopathological observations*

All rats in the three groups were fasted for 24 h before the experiment and had free access to water. After treatment, rats were anaesthetized using pentobarbital sodium and blood was collected through the orbital venous plexus at 0, 7, 14 and 30 days. Blood samples were centrifuged at 3000r/min for 5min. Concentration of E2, P and PRL in serum was measured. Hormone concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the procedures recommended by the manufacturer (Shang Hai Heng Yuan Biological Technology Co., Ltd, China).

Mammary gland tissues from all groups were fixed in 10 % buffered formalin, embedded in paraffin,

sectioned into 4 μm pieces, stained with Haematoxylin-Eosin (H&E) and examined using optical microscopy.

*Real-time quantitative PCR*

Tissues were obtained from rat mammary glands and total RNA was extracted with Trizol reagent (Shanghai Sangong Biotech Co., Ltd. Shanghai, China). Total RNA, OligodT (15), 5×Reaction buffer, dNTPs, ribonuclease inhibitor and reverse transcriptase were successively added into the sterile enzyme-free Eppendorf tubes. Reverse transcription was performed at 42 °C for 30 min and then 70 °C for 5 min. The cDNA was synthesized by a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc, NY, USA). Reaction conditions for synthesis were: initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s followed by a final extension at 60 °C for 5 min (Table I).

**TABLE I** - Sequences of primers used in gene expression analysis

Gene	Forward and reverse primers
(estrogen receptor) ER	F:ACTCGCTACTGTGCTGTGTGT R:TCGGCGGTCTTTTCGTATCCC
(progesterone receptor) PR	F:TGCTGACCAGTCTCAACCAAC R:TGGTAAGGCACAGCGAGTAGA
(prolactin receptor) PRLR	F:AACAAGCCCAGAAAGTCCCTC R:GCAGGCACCGAATGTTGTTAT
GAPDH	F:TTCCTACCCCAATGTATCCG R:CATGAGGTCCACCACCTGTT

#### Western blot

Total protein was extracted from the mammary glands and the concentration was determined using Bradford method. 40 µg of total protein per sample was used for SDS-PAGE electrophoresis and using the following parameters: 5 % concentration gel, 75 V for 20 min; 10 % separation gel, 120 V for 60 min. The protein was then transferred to Poluvinylidene Fluoride (PVDF) membranes (Millipore, USA). The membranes were incubated overnight with anti-β-actin (1:500), estrogen receptor (ER) (1:2000) and prolactin receptor (PRLR) (1:2000) rabbit polyclonal antibodies (Santa Cruz Biotechnology, CA, USA), and progesterone receptor (PR) (1:800) rabbit polyclonal antibodies (Abcam plc, Cambridge, UK) in 5 % milk/TBST at 4 °C. Membranes were washed five times with TBST and incubated with horseradish peroxidase-conjugated antibody for 1 h at room temperature. Western blot analysis was performed using enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc, NY, USA) and exposed to Kodak radiographic film. Images were then acquired using gel imaging system and analysis was performed with Alpha software system.

#### Statistical analysis

All data were statistically analyzed using SPSS 16.0 software and expressed as mean ± standard deviation ( $\bar{x} \pm s$ ). Inter-group comparison was performed using *t* test. *P* < 0.05 or *P* < 0.01 indicated that the difference was statistically significant.

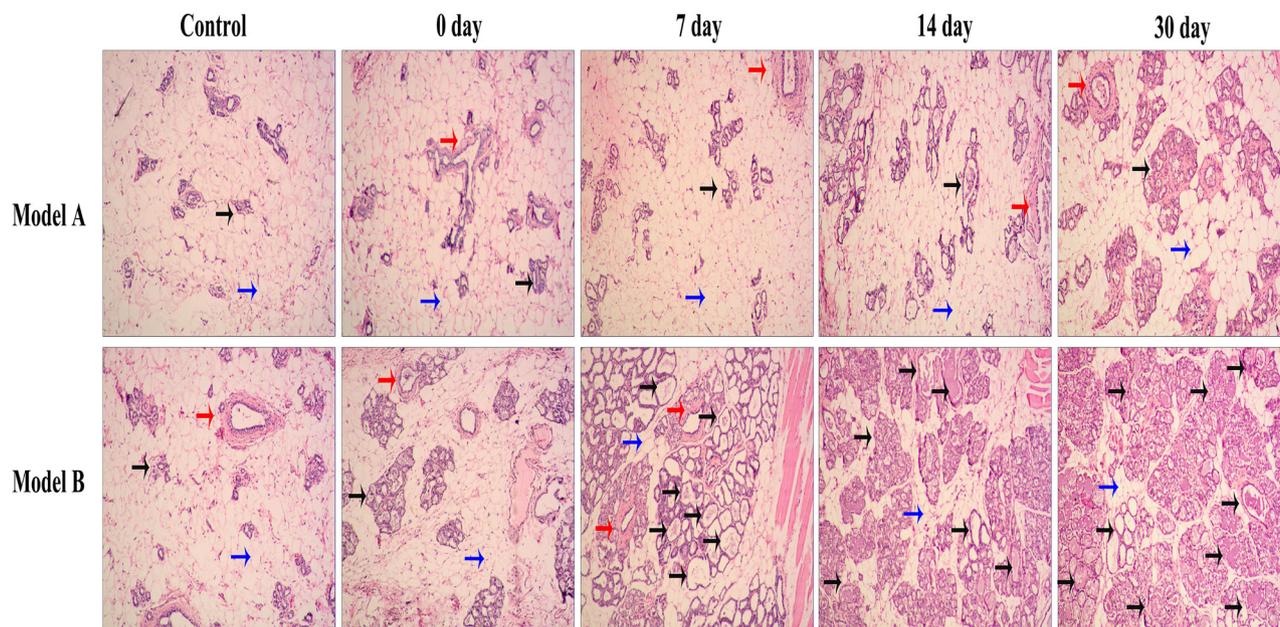
## RESULTS AND DISCUSSION

### Results

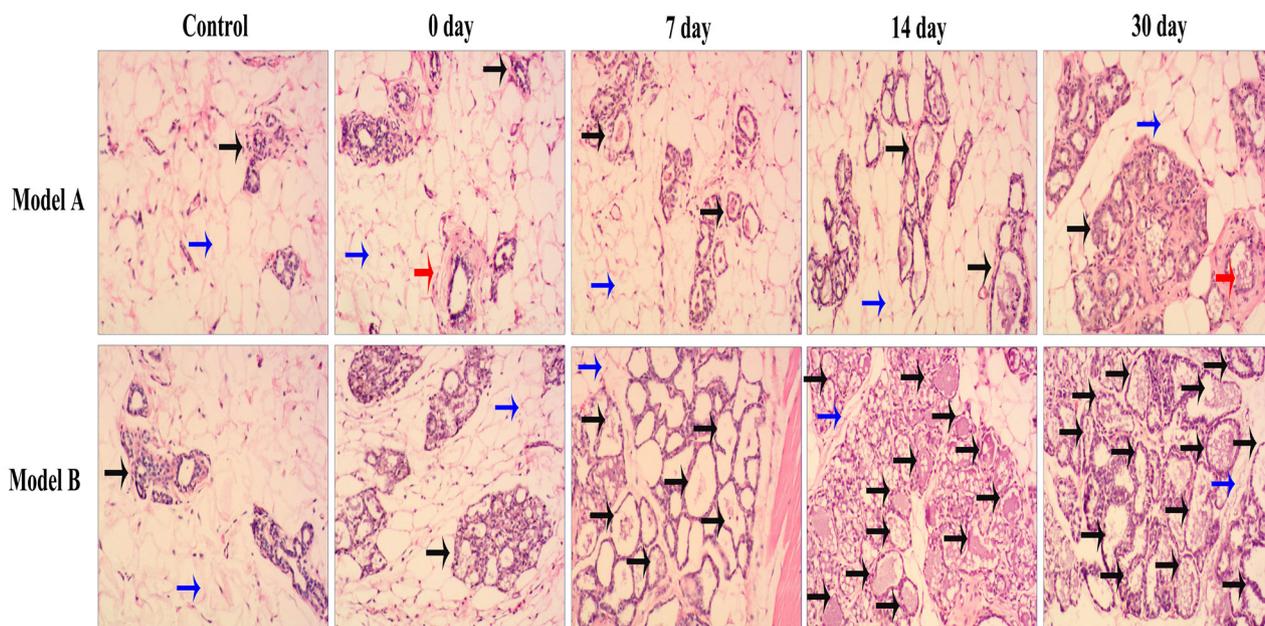
*Morphological and pathological changes were observed in rat mammary gland tissue*

The degree of MGH in models A and B were compared. Mammary gland (MG) proliferation of the control group was quiescent (Figure 2A and 2B). MG lobules had abundant scattered connective tissue either without or with small acini and ducts with no secretory products. No significant differences were observed between MG tissues of model A group and those of the control group on days 0 and 7. Partial acini of the MG lobules and ducts in model A were slightly larger on days 14 and 30 with minor secretions present compared with those of the control group.

In model B, the acini of the MG lobules and ducts were mildly enlarged on day 0 with fewer secretions compared with the control group. On day 7, a small number of secretory products was observed in the acini of the MG lobules, which were significantly larger in size compared with those of the control group. In this model, the connective tissue decreased significantly. Notably, the number of acini in the MG lobules of model B were significantly higher number and larger with increased secretions on days 14 and 30 compared with the control group. At these time points, little connective tissue was present due to increase in size and number of acini within the MG lobules. Overall, these results showed that model B MGH was more extensive compared with that of model A (Figures 2A and 2B).



**FIGURE 2A** - Histological HE straining analysis on day 0, 7, 14 and 30 after model replicate cycle in model A and B (black arrowhead: lobuli glandulae mammariae; blue arrowhead: connective tissue; red arrowhead: duct; original magnification, 40×, Olympus, Beijing, China).

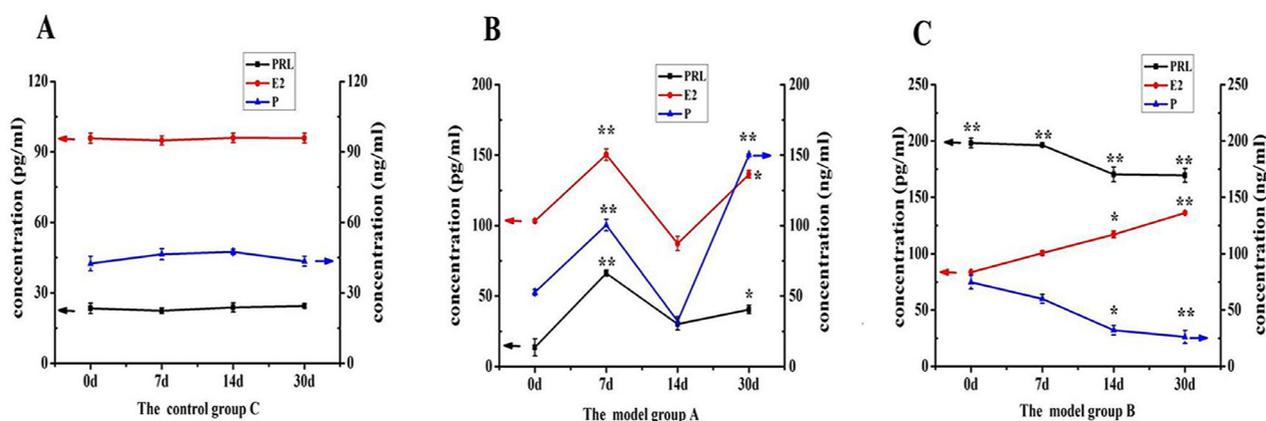


**FIGURE 2B** - Histological HE straining analysis on day 0, 7, 14 and 30 after model replicate cycle in model A and B (black arrowhead: lobuli glandulae mammariae; blue arrowhead: connective tissue; red arrowhead: duct; original magnification, 100×, Olympus, Beijing, China).

### Determining E2, P, and PRL concentrations in rat serum

The control group (C) showed constant concentrations of E2, P, and PRL over the experimental period; however, E2 concentrations were significantly higher compared with PR and PRL concentrations (Figure 3A). E2, P, and PRL concentrations in model A rats varied as shown in Figure 3B. At the start of the experiment, the concentrations increased, then decreased, and finally increased toward the end of the experiment. E2, P, and PRL levels were significantly higher in the serum of model A rats on days 7 and 30 ( $P < 0.05$  and  $P < 0.01$ , respectively) compared with the control group (C). However, the levels were not significantly different after 14 days ( $P > 0.05$ ). Interestingly, the three hormone levels showed different trends in model B compared with those of model A. In model B, PRL

and PR concentrations decreased at the beginning of the study. However, PRL levels were significantly higher in model B throughout the study period ( $P < 0.01$ ) compared with the control group. On the contrary, P levels were significantly lower in this model compared with those of the control group on days 14 and 30 ( $P < 0.05$  and  $P < 0.01$ , respectively). Notably, E2 concentration increased at the beginning of the study and were highest on days 14 and 30 compared with those of the control group ( $P < 0.05$  and  $P < 0.01$ , respectively). These results imply that E2, P, and PRL hormones are released in a more steady-state in model B rats compared with that of model A rats. Further, PRL concentrations were higher in model B rats throughout the study period compared with those of model A rats, indicating that model B is a more suitable rat model to study the relationship among the three hormones and MGH.



**FIGURE 3** - Comparison of serum sex hormone (E2, P and PRL) concentrations between model A and model B. (A) Normal control group; (B) Model A; (C) Model B. Data are presented as mean  $\pm$  SD values. \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. normal control group.

### Real-time quantitative PCR detection of estrogen receptor (ER), progesterone receptor (PR) and prolactin receptor (PRLR) mRNA expression

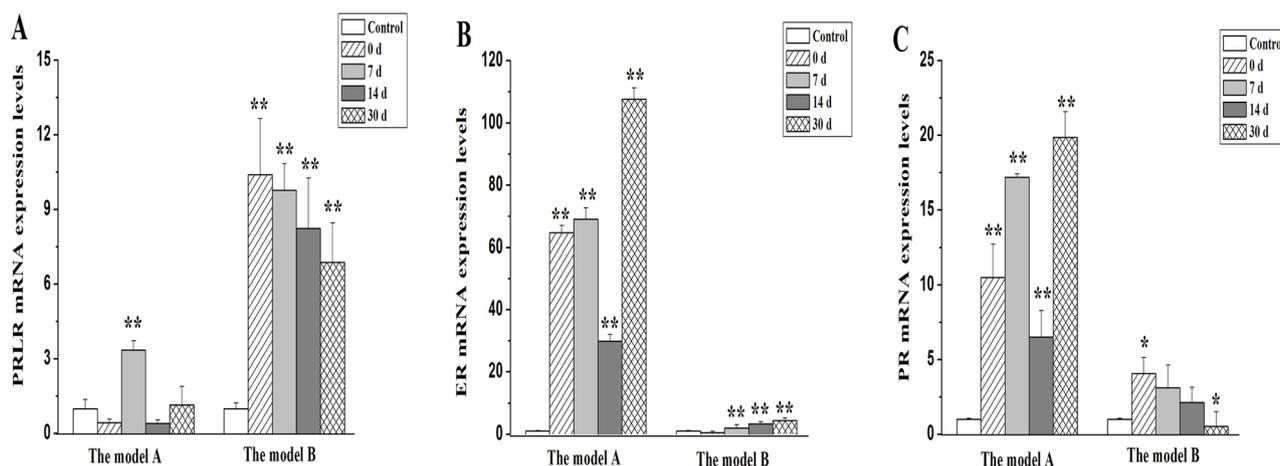
PRLR mRNA expression level in model A rats at day 0, 14, and 30 was not significantly different compared with the control group ( $P > 0.05$ ). However, in model A, PRLR mRNA expression level at day 7 was significantly higher compared with that of the control group ( $P < 0.01$ ). ER and PR mRNA expression in model A was

significantly higher compared with those of the control group on throughout the experiment ( $P < 0.01$ ) (Figure 4B and C). Notably, ER, PR, and PRLR expression levels showed no regular trend in model A.

PRLR mRNA expression levels in model B showed a gradual decrease (Figure 4A). However, PRLR mRNA expression level in model B was higher compared with that of the control group throughout the study period ( $P < 0.01$ ). Interestingly, ER mRNA expression this model showed increased gradually compared with that of the

control group. Further, ER mRNA expression level in model B was significantly higher on days 7, 14 and 30 compared with the level in the control group ( $P < 0.01$ ). Notably, PR and PRLR mRNA expression levels showed a similar decreasing trend over the study period. PR mRNA expression level in model B on day 0 was higher compared with that of the control group ( $P < 0.01$ ). On the contrary, on day 30 the level was significantly lower

compared with that of the control group ( $P < 0.01$ ). No significant differences in PR mRNA expression levels were observed for model B and the control group on days 7 and 21 ( $P > 0.05$ ). The results showed that the expression patterns of genes for the three receptors in model B rats were positively correlated with changes in E2, PR, and PRLR concentrations and the progressive changes in clinical disease status.



**FIGURE 4** - mRNA expression levels of PR, ER and PRLR in model A and B. (A) PRLR, (B) ER, (C) PR. Data are presented as mean  $\pm$  SD values. \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. normal control group.

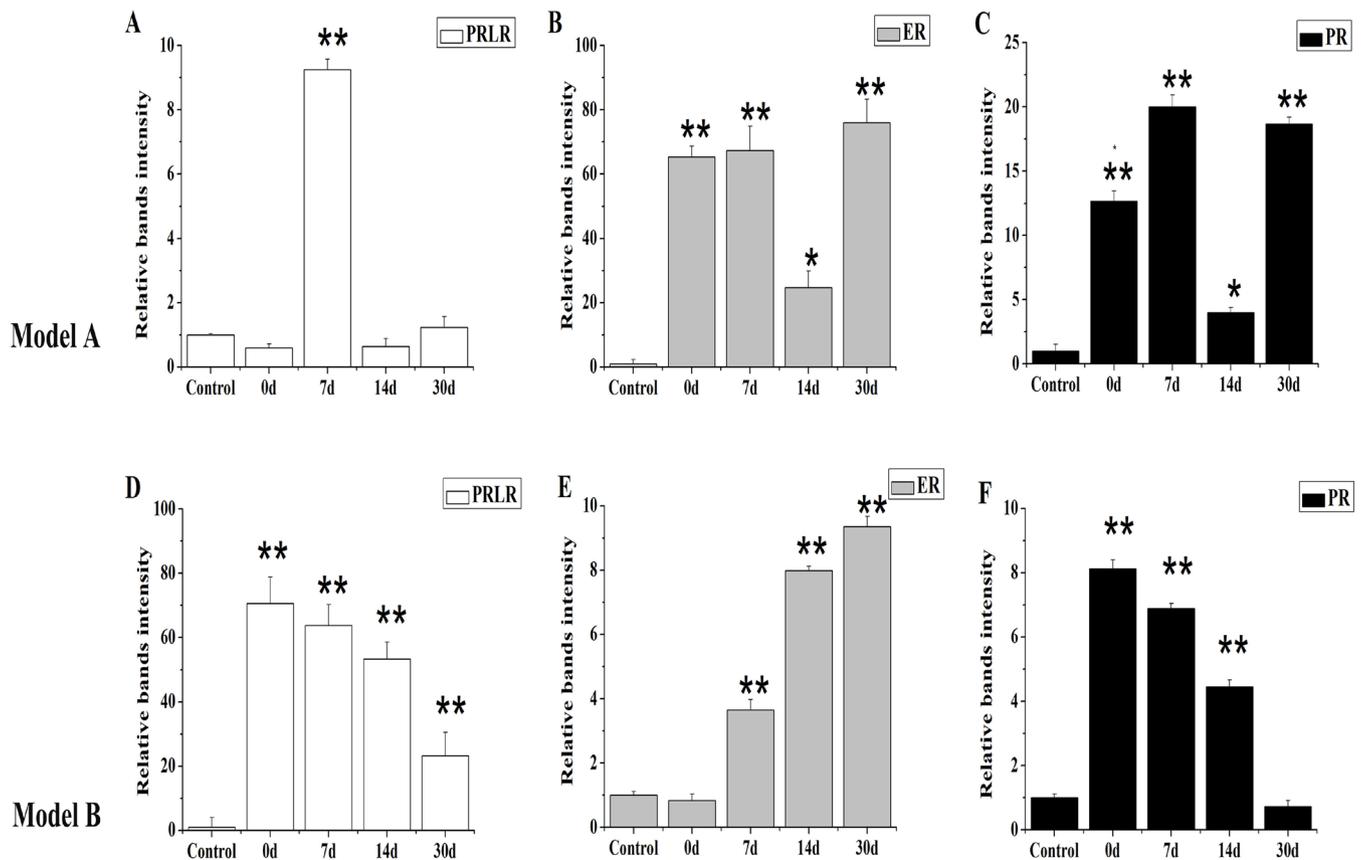
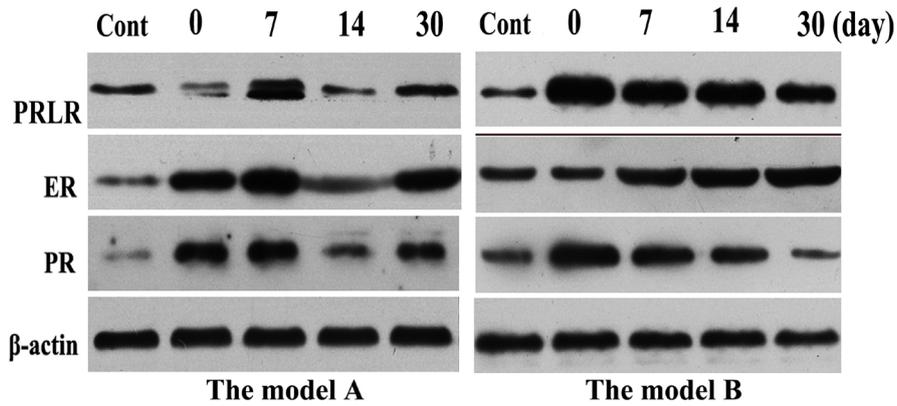
#### ER, PR, and PRLR protein expression levels in mammary gland tissue

Figure 5 showed the protein levels of PRLR, ER and PR in model A and model B. ER, PR, and PRLR protein expression levels in model A showed no consistent trend, similar to the observation reported for mRNA expression levels of the three receptors. On day 7, PRLR protein expression level in model A was 9.6-fold ( $P < 0.01$ ) higher compared with the control group but was not significant different on the other days ( $P > 0.05$ ). ER protein expression level were 42-, 42.6-, 14.6-, and 52.6-fold higher on days 0, 7, 14 and 30 respectively, in model A compared with those of the control group ( $P < 0.01$ ). In model A, PR protein expression levels on days 0, 7, 14 and 30 were 11.3-, 18-, 4.8-, and 16.5-fold higher, respectively, compared with those of the control group ( $P < 0.01$ ).

ER, PR, and PRLR protein expression levels in model B rats showed a consistent trend. ER protein expression level showed an increasing trend with time, while PRL and PR protein expression showed a decreasing trend over the study period. In model B, PRLR protein expression levels on days 0, 7, 14 and 30 were 40.2-, 35.5-, 30.4-, and 12.8-fold lower, respectively, compared with those of the control group ( $P < 0.01$ ). On the contrary, ER protein expression levels on days 7, 14 and 30 were 2-, 4- and 4.05-fold higher, respectively in model B, compared with those of the control group ( $P < 0.01$ ). However, ER protein expression on day 0 was statistically comparable to that of the control group ( $P > 0.05$ ). PR protein expression levels on days 0, 7 and 14 were 8.3-, 5.4-, and 3.97-fold lower, respectively in model B, compared with the control group ( $P < 0.01$ ). However, PR protein expression on day 30 was statistically comparable to that of the control group ( $P > 0.05$ ). The results showed that the

protein expression levels of the three receptors in model B rats were positively correlated with changes in the

corresponding hormone concentrations and expression levels of the receptor genes.



**FIGURE 5** - Protein levels of PRLR, ER and PR as determined using Western blot assays in mammary gland tissues of model A and model B. Quantitation of western blot results. (A) PRLR, (B) ER and (C) PR protein levels in model A group, (D) PRLR, (E) ER and (F) PR protein levels in model B group. Data are presented as mean  $\pm$  SD values. \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. normal control group.

## DISCUSSION

HPRL and MGH are common diseases of the endocrine system that immensely affect women health. Previous studies report an overall increased cancer risk in patients with both HPRL and MGH (Berinder *et al.*, 2011; Cortez *et al.*, 2014). However, etiologies of MGH and HPRL are not well known, therefore, currently effective therapies are limited (Chen *et al.*, 2015). The balance of sex hormones is positively correlated with normal function of the hypothalamus-pituitary gland-ovary-axis. Previous studies report that E2, P, and PRL hormones play a key role in mammary gland pathologies, including HPRL and MGH (Silva-Alves, Barcelos Filho, Franci, 2017; Traslaviña *et al.*, 2011). For instance, PRL is involved in mammary gland development, lactation and corpus luteum maintenance, by binding to PRLR through the mediation of different signaling pathways (Ben-Jonathan, LaPensee, LaPensee, 2008; Ormandy *et al.*, 1997). E2 enhances ER levels and promotes high expression of ER $\alpha$  in atypical hyperplasia and atypical ductal hyperplasia. In addition, E2 is a crucial mitogen that boosts breast cell proliferation and clonal expansion (Shoker *et al.*, 1999; Ding *et al.*, 2006). Low PR expression levels, as in typical nuclear transcription, is an indicator of triple-negative breast cancer (Zhao *et al.*, 2019). Previous studies report higher E2 and PRL concentrations in MGH model induced with estradiol benzoate and progesterone, whereas PR concentrations were lower compared with those in the control group (Wang *et al.*, 2013a). However, lower E2 and P concentrations are observed, whereas higher PRL concentrations are observed in HPRL model induced with metoclopramide dihydrochloride compared to the concentrations in the control group (Wang *et al.*, 2013b). These findings imply that the three hormones play important roles in regulation of MGH and lactation. However, changes in the expression levels of E2, P, and PRL in HMG and HPRL models were not significant. Therefore, it is important to explore a suitable animal model for studying the relationship among MGH, HPRL and the hormone levels.

Mammary gland hyperplasia and neoplasia have characteristic morphology, phenotype, and hormone receptor expression profiles for E2, P, and PRL hormones

(Ellis, 2010). In the present study, features of model B were more comparable to classical MGH compared with those of model A. In model B, MGH was characterized by significant increase in acini of mammary gland lobules, expansion of acini and ducts, superfluous secretions, and scarce connective tissue compressed by the acinar expansions (Figure 2A and 2B).

E2, P, and PRL hormones play an important role in mammary gland development and lactation. Further, PRL and E2 synergistically activate estrogen target genes and promote breast cancer cell proliferation (Rasmussen *et al.*, 2010). In addition, PRL improves sensitivity of E2 in mammary tumors of mice (Arendt *et al.*, 2011). A previous study reports that PR and ER- $\alpha$  promote regulation of target gene transcription through hormone interactions (Zhao *et al.*, 2019). These studies suggest that the three hormones act synergistically. In this study, analysis of model B experimental results showed that subsequent supplementation of E2 and P may have led to increase in P levels, resulting in the inhibition of E2. Therefore, E2 concentrations in model B were lower compared to those of the control group at day 0. However, high PRL concentrations in the early stages of the experiment gradually inhibited P secretion; therefore, inhibiting sustained antagonism and allowing E2 concentrations to increase gradually. Similar findings were reported by Petraitiene *et al.*, (2001).

Notably, E2, P, and PRL levels in model B rats were more steady compared with the levels in model A rats. Further, PRL concentrations were significantly higher compared with the concentration in the control group (Figure 3C). In addition, the corresponding mRNA and protein expression levels of the three hormones in model B were consistent with the hormone concentration changes with a consistent trend. However, the degree of MGH in model A rats was milder compared with the model B, and E2, P, and PRL concentrations showed inconsistent trends (Figure 3B).

Therefore, the method used for model B is more effective in establishing a rat model with stable pathologic signs of MGH with HPRL. The three hormones indices in model B showed steady changes and greatly simplified the duplicative process involved in animal model development. This, in turn, reduced the number of

animals required in the preliminary stages of the study. A similar study reports a systematic evaluation of the scientific approach to provide an animal model that is suitable for studying various diseases (Guo *et al.*, 2015).

To understand the interaction between MGH and HPRL, this study replicated these two disorders in rats for the development of an animal model. In this animal model, typical clinical symptoms related to the two disorders should be reflected, and changes in the indices detected should be observed. This model should also have a long clinical process as seen with the MGH process in people (Lv *et al.*, 2016). In addition, HPRL should occur relatively fast (a prolactin level > 25 ng/mL could indicate HPRL) (Schlechte *et al.*, 2003). The rat model produced in this study met the requirements for follow-up studies.

In this study, we were unable to scientifically define normal values or ranges of the three hormones, due to rapid physiologic responses and challenges in determining boundaries between each time point in the rat models. Further, we were not able to calculate proportions of the hormones as it is done in clinical setups (Bo *et al.*, 2016). Therefore, mutual influence of the three hormones was elucidated using the changes in hormonal trends. The findings of this study show that a balance exists among the three hormones. Changes in concentration of one hormone resulted to changes in the levels of the other two hormones. Notably, hormonal imbalances results in typical clinical symptoms of the disease (Chuffa *et al.*, 2017; Gupta *et al.*, 2017).

## CONCLUSION

The group B rat model established in this study showed clinical symptoms related to MGH and HPRL, therefore, it can be used to study association of E2, P, and PRL hormones and the two conditions. However, further research should be carried out to explore signal transduction mechanisms associated with the three hormones.

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## CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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