Therapeutic effects of total alkaloids of *Fructus Hordei Germinatus* in hyperprolactinemis rats

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Abstract: *Fructus Hordei Germinatus* is widely used in treating hyperprolactinemia as a kind of Chinese traditional herb in China. However, its active composition of curing hyperprolactinemia remains unclear. This study investigates the activity of total alkaloids of *F. H. Germinatus* (AFH) in hyperprolactinemia rats. High-dose, middle-dose and low-dose AFH were administered into the stomach of hyperprolactinemia rats for 30 days. It revealed that high-dose AFH had obvious curative effect in treating hyperprolactinemia. It could regulate serum E2, P, PRL, FSH, LH levels to normal, decrease the pituitary prolactin positive cell number, mRNA expression level and inhibit the hyperplasia of mammary gland in hyperprolactinemia model rats effectively. The *F. H. Germinatus* contained total alkaloids 42. 74±0. 08mg hordenine equivalent (HE)/g the sample using acid dye colorimetry method. *F. H. Germinatus* should be developed as an anti-hyperprolactinemia product deeply.

Keywords: *F. H. Germinatus*; total alkaloids; hyperprolactinemia; hyperplasia of mammary gland.

INTRODUCTION

Hyperprolactinemia, which is one of the most common endocrine disorders of the hypothalamus-pituitary axis (PRL>25ng/ml) in young women, is associated with galactorrhea and ovulatory dysfunction that results in menstrual irregularities and barren (Lee *et al*., 2012).

Hyperprolactinemia can occur at any age, and the prevalence varies from 0. 4% in the normal adult population to as high as 9-17% in women with menstrual problems such as amenorrhea or polycystic ovarian syndrome (Biller 1999; Greer 1980). Typical examples which induce hyperprolactinemia are hypothalamus-pituitary lesions pituitary tumor severe liver or kidney disease neuritis or irritations of the spinal cord depression or other physiological factors such as pregnancy and lactation (Lutz, 2012; Yu-lee 1998). Galactorrhea is a common kind of female disease induced by hyperprolactinemia. Chemical drugs are used in treating them, but they always bring many side effects such as menstrual disorder and the relapse rate is very high.

Bromocriptine and cabergoline are effective in curing hyperprolactinemia, but 12% patients can not endure bromocriptine (Webster *et al*., 1994), and the expense of them is very expensive (Mah, 2002).

Fructus Hordei Germinatus (fig. 1) is a kind of herb germinating from barley widely used in China. In the ancient book (Zhonghuazi) of Song Dynasty, it was used to treat female galactorrhea effectively (Wang Xiong *et al*., 2010). In China, doctors always use decoction of *F. H. Germinatus* for galactorrhea patients, and have achieved curative effect without toxic side effect. (Zhu Dong-qing, 2010; Guo Xiao-dong, 2006). It has been reported that the *F. H. Germinatus* extract could decrease prolactin level in female hyperprolactinemia mice (Wei An-hua *et al*., 2009; Zhou Wei *et al*., 2008). However, the active composition of *F. H. Germinatus* in treating hyperprolactinemia is not reported yet. In this study, we evaluated the anti-hyperprolactinemia activity of AFH by serum hormone levels, pituitary prolactin positive cell number and mRNA expression and pathomorphology observation of mammary gland tissue in rats. And the total alkaloids content of *F. H. Germinatus* was also determined.

MATERIALS AND METHODS

Plant material and extraction

The herbal samples of *F. H. Germinatus* were collected from Bozhou, Anhui in July 2012. Taxonomic identification of the plant was performed by Prof. Dr. Jin hu Wu. Samples of *F. H. Germinatus* were extracted with 90% ethanol for one hour. After two times, filtrate it and concentrate until extractum. Add water into the extractum, filter, concentrate and dry into thick powder. Afterwards, percolate the powder with 6 times 5% HCL solution, then add NaOH into it to regulate PH to 10 and was set for 24 h. After filtered, concentrated and dried, we get the rude sample. Finally, dissolve it with ether, extracted by 1% HCL. Then regulate PH of the extract liquor to 10 by ammonia. Put up for the night, and wash the sediment for two times with water. Dry it in 70oX to gain white powder. The powder was recrystallized by methanol-acetone, then we obtained the colorless granulated pure product of total alkaloids (weight of total alkaloids/weight of raw materials=1.37%, g/g).

Metoclopramide was gained from Xuzhou Laien

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pharmaceutical Co., Ltd. Bromocriptine methanesulfonate was obtained from Novartis Pharma Schweiz AG. Other reagents were analytical pure grade.

Fig. 1: *F. H. Germinatus*

Animals

Female Wistar rats weighing 200-220g were obtained from Hubei Center for Diseas Control and Prevention, Wuhan, Hubei. The animals had free access to food and water and were allowed to acclimatize for at least one week before use. All experiments were approved by the Animal Care and Use Committee and were carried in compliance with the Animal Welfare Act and the NIH guidelines (NIH publication No. 80-23, revised 1996).

Determination of total alkaloids content

Total alkaloids were determined using acid dye colorimetry method (Zeng Bao *et al*., 2012). The total alkaloids (600μg/mL) or standard hordenine ethanol solution (10-80μg/mL), 1. 0 mL was mixed with 2. 0mL of phosphate buffer solution (pH=7) 1.0mL of bromothymol blue solution (1% w/v) and 3. 0mL of chloroform. The mixture was put at room temperature (27±2°C) for 1 min with shaking, then static for 60 min. Get the chloroform layer. The absorbance of the mixture was measured at 284nm using a UV-vis spectrophotometer (SHIMADZU, Japan). The content of total alkaloid compounds was calculated as mean±SD (n=3) and expressed as milligrams of hordenine equivalent (HE) in 1 g of the *Fructus Hordei. G* sample.

Preparation of model rats and Treatments

The hyperprolactinemia rat model was performed as previously described with a little modification (K. C. Lin 1988). Metoclopramide dihydrochloride injection was subcutaneous injected at the back of rats according to 50mg/kg body weight. Experiment was performed at 9:00 and 15:00 respectively every day for five days.

All rats were divided into six groups of ten individuas: Control, model group, model plus positive drug bromocriptine group, plus high-dose AFH (898. 0mg/kg), plus middle-dose AFH (449. 0mg/kg) and plus low-dose AFH (224. 5mg/kg). Each dose was dissolved in a 2 ml water, and administered through the mouth to the stomach by a syringe. The dosage was calculated from the daily human AFH clinical dosage based on body surface area. Control and model rats received 2 ml of water. All group mice were intragastric administrated for 30 days.

Serum E2, P, PRL, FSH and LH determination

At the end of 30 days, serum E_2、P、PRL、FSH、LH of all rats were measured. All measurement were used by ELISA kits provided by Shanghai lichen Inc. (China).

Pituitary prolactin positive cell number analysis

Embed the rat pituitary tissue with paraffin wax. After dewaxing, hatch for 5min with 3% H_2O_2 at room temperature, then add the rat PRL antibody (first antibody, 1:300) for 60min at room temperature, and wash it with PBS for three times. Then add Envision reagent (including secondary antibody and third antibody) into it, hatch for 60min at room temperature, and wash it with PBS for three times. Use DAB solution for coloration, then wash it with distilled water, redye, dehydration and finalize to analysis PRL positive cell numbers by Microsoft (×200 times).

Pituitary prolactin mRNA expression level determination

Put the pituitary tissue into glass grinder with 1mL Trizol solution, homogenyate was added into 1. 5mL Eppendorf tube, and lay up for 5min at room temperature. Add 0. 25mL chloroform into it, shake for 15 seconds, and lay up for 3min at room temperature. Centrifugate (12000g) for 15min in 4oX. The upper aqueous phase was added with equal volume isopropyl alcohol in 1. 5mL Eppendorf tube, and lay up for 10min at room temperature. Centrifugate (12000g) for 10min in 4oX. Throw away the supernatant, and add 0. 9mL 75% ethanol into it. Centrifugate (10000g) for 5min in 4oX. Repeat it. Throw away the supernatant and dry it at room temperature, then 30-50μl no RNA enzyme water was added into it for dissolving the RNA sample.

Put the RNA sample and primer into PCR in 95°C for 3min, then 5μl 5×Buffer, 5μl dNTP, 1μl RNA enzyme inhibitor, 1μl M-MLV reverse transcriptase was added into it successively in ice-bath. Inactivate the reverse transcriptase in 42°C for 60min, then 95°C for 2min. PCR amplification system: PCR-Mix 12. 5μl, 2μl rat prolactin primers, 2μl internal primer, 2μl reverse transcription product, no RNA enzyme water, centrifugal and mixed, then add a little sterile liquid paraffin into it for 3min in 94°C. Go to next circle: 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 60 seconds, 23 circles together. The last circle extended to 8min at 72°C. After the amplification progress, put the amplification products on 1. 5% agarose

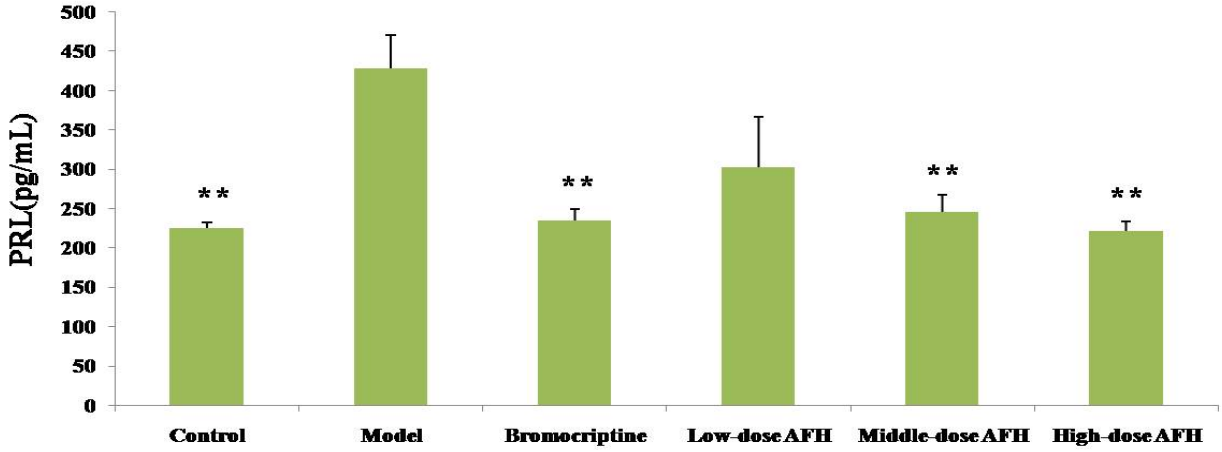

Fig. 2: Serum PRL concentration in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

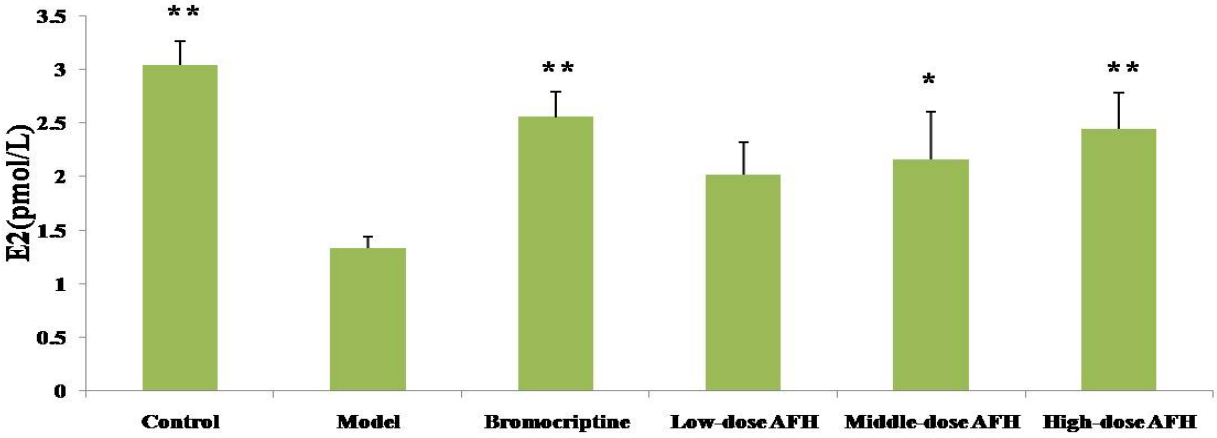

Fig. 3: Serum E2 concentration in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

gel for 20min 70V constantoltage electrophoresis, then determine the gray value of sample using UVP computer image analysis system. With β-actin as internal control, count the relative amount of pituitary prolactin mRNA expressions.

Histological analysis

Tissues of mammary gland from each group were fixed in 10% formalin solution, embedded in paraffin, sectioned into 4μm thickness, stained with Hematoxylin-Eosin (H-E) and Masson-Trichrome (M-T) and examined using optical microscopy. The severity of mammary gland hyperplasia was based on four parameters (hyperplasia of gland alveolus, lobule, shape and thickness of vessel and secretion).

STATISTICAL ANALYSIS

Values were expressed as the means ±SEM. Significant differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by a two pairs Student's t-test. $P<0.05$ was considered statistically significant.

RESULTS

Total alkaloids content of fructus hordei germinatus sample

The *F. H. Germinatus* contained total alkaloids 42.74±0.08 mg hordenine equivalent (HE)/g the sample.

Effect of AFH on serum E_2, P, PRL, FSH and LH levels

The serum PRL ($P<0.01$ vs blank control) concentrations increased significantly, E_2, P and FSH ($P<0.01$ vs control) and LH ($P<0.05$ vs control) concentration decreased in hyperprolactin-emia model rats. Such an increased PRL was significantly attenuated by treatment with bromocr-iptine、middle-dose and high-dose AFH afer one month of administration ($P<0.01$ vs the model group). Bromocriptine has obvious curative effect in E2 ($P<0.01$ vs model), P ($P<0.05$ vs model),

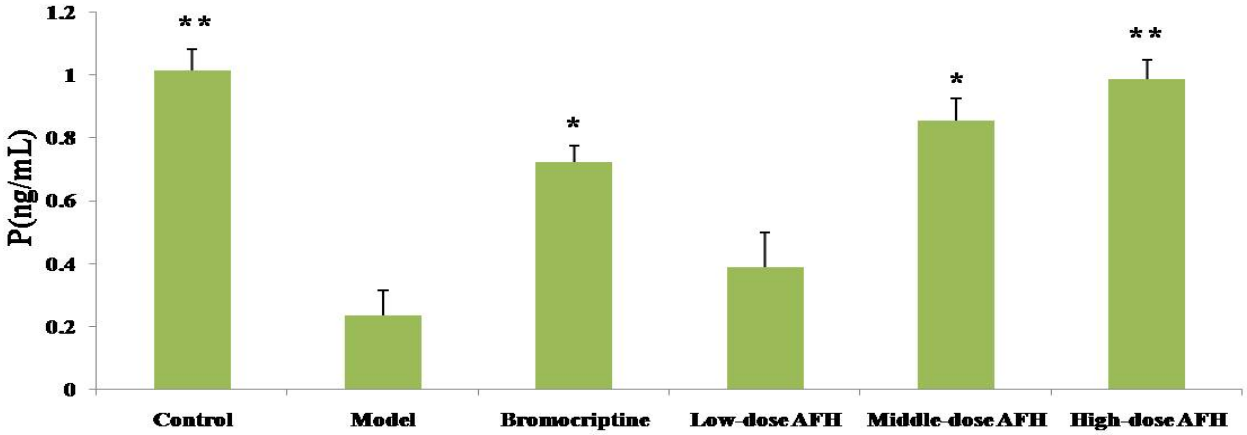

Fig. 4: Serum P concentration in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

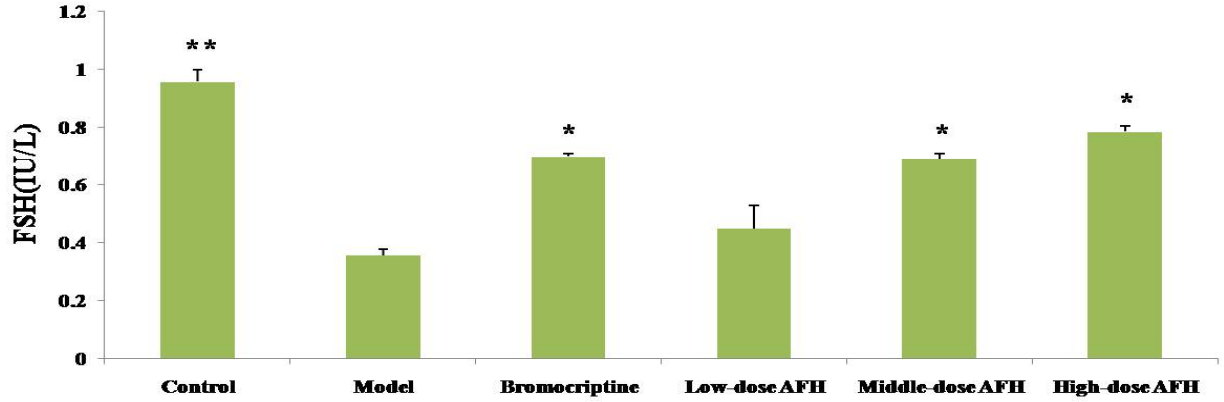

Fig. 5: Serum FSH concentration in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

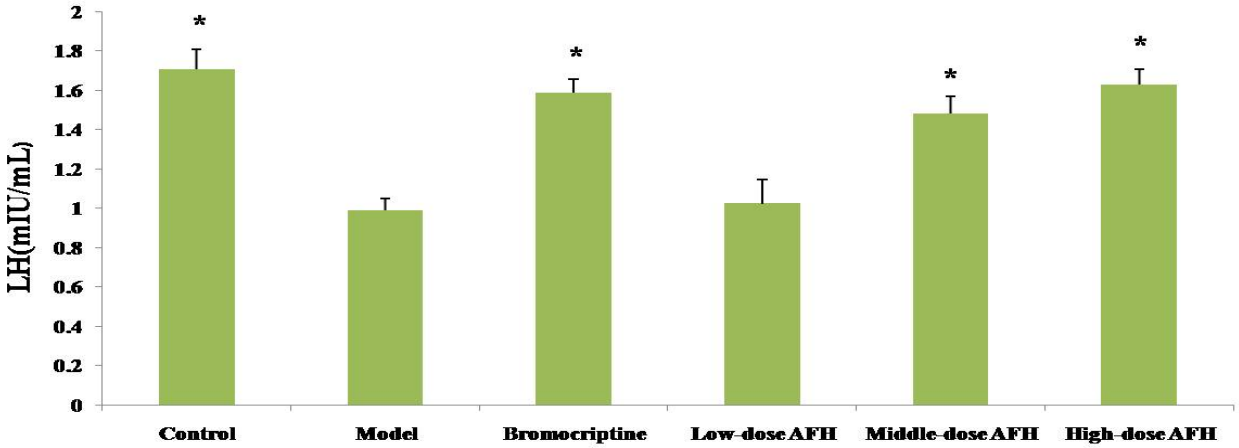

Fig. 6: Serum LH concentration in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

FSH ($P<0.05$ vs model), LH ($P<0.05$ vs model). And E2 ($P<0.01$ vs model), P ($P<0.01$ vs model), FSH ($P<0.05$ vs model), LH($P<0.05$ vs model) were regulated by high-dose AFH obviously (figs. 2-6). The low-dose AFH had no obvious curative effect in treating hyperprolactinemia.

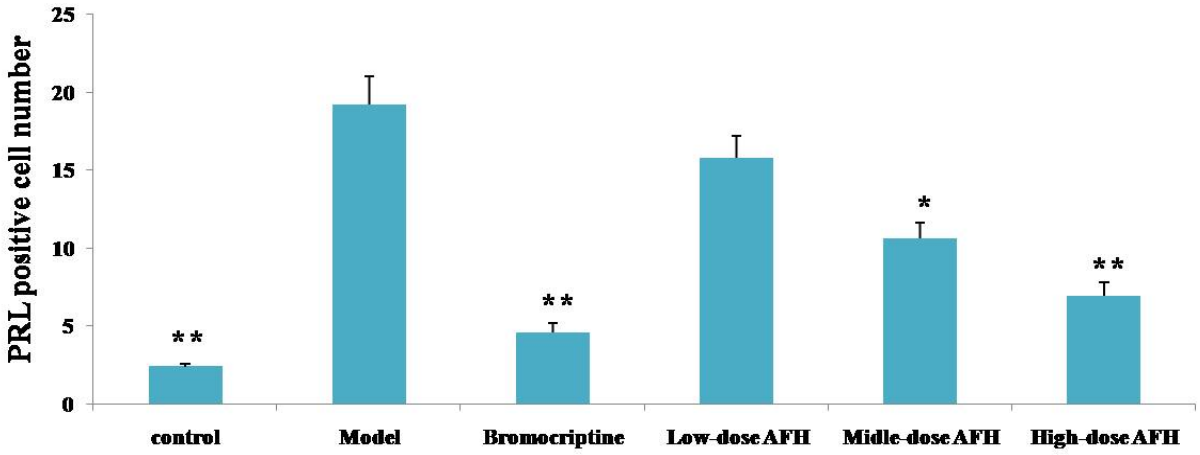

Fig. 7: Pituitary prolactin positive cell numbers in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

Effect of AFH on pituitary prolactin positive cell number

As shown in fig. 7, the pituitary prolactin positive cell number of model group increased significantly compared with control group ($P<0.01$). It was attenuated by treatment with bromocriptine ($P<0.01$ vs "model" control), middle-dose AFH ($P<0.05$ vs "model" control) and high-dose AFH ($P<0.01$ vs "model" control) afer one month of administration.

Effect of AFH on pituitary prolactin mRNA expression level

The PRL mRNA expression level of model rats is significantly higher than control rats ($P<0.01$). It was attenuated by treatment with bromocriptine ($P<0.01$ vs model), middle-dose AFH ($P<0.05$ vs model) and high-dose AFH ($P<0.01$ vs model) afer one month of administration (fig. 8-9).

Effect of AFH on pathomorphology of mammary gland tissue

Compared with control group, hyperplasia of lobules and gland alveolus and much secretion was found in the mammary gland of hyperprolactinemia model rats (fig. 10B). In bromocriptine-treated group at week 4, the hyperplasia of lobules and gland alveolus of mammary gland lessened, secretion and lymphocyte decreased in intracavitary (fig. 10C). The mammary gland of groups given high-dose and middle-dose AFH recovered well. There was no hyperplasia in gland alveolus and lobules of mammary gland, secretion and lymphocyte was seen (fig. 10D and E). Much gland alveolus and lobules hyperplasia was seen in the low-dose AFH yet (fig. 10F). This illustrated that AFH could inhibit the hyperplasia of mammary gland in hyperprolactinemia model rats effectively.

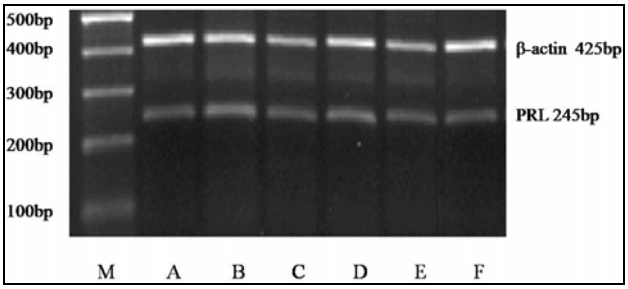

Fig. 8: Pituitary prolactin mRNA expressions by RT-PCR in different groups. M: Marker; A: Control group; B: Model group; C: Bromocriptine group; D: Low-dose group; E: Midle-dose group; F: High-dose group.

DISSCUSSION

Hypothalamus-pituitary-gonadal axis plays an important part in the maintenance of normal physiologyical body function and homeostasis of the internal environment. (R. G. Goya1991). When extraneous factors act on human body, and sex hormone secretion becomes abnormal, thus leads to many kinds of disease such as hyperprolactinemia, hyperplasia of mammary gland (HMG), hysteromyoma, infertility and so on. In mammals, PRL can regulate mammogenesis promote milk production, initiate and sustain lactation through autocrime and paracrime as a kind of cell factor (Freeman ME 2000; Kelley KW 2007). PRL has many functions on breast tissue including uridine conversion, influences on milk protein synthesis and mammary cell sodiumt-ransport, incorporation into DNA, and breast fatty acid synthetase activity (Malarkey WB *et al*. 1977).

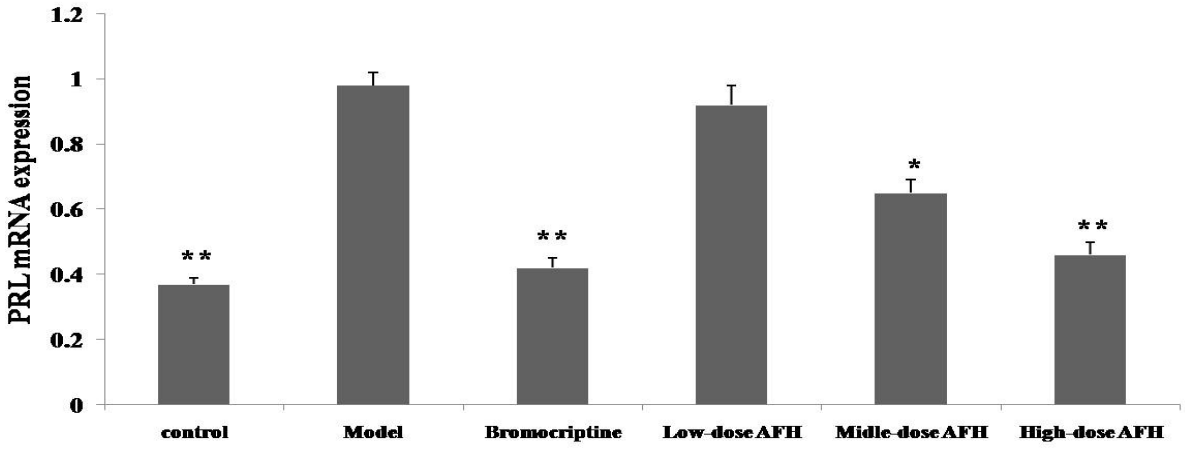

Fig. 9: The relative amounts of pituitary prolactin mRNA expression levels in different groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group.

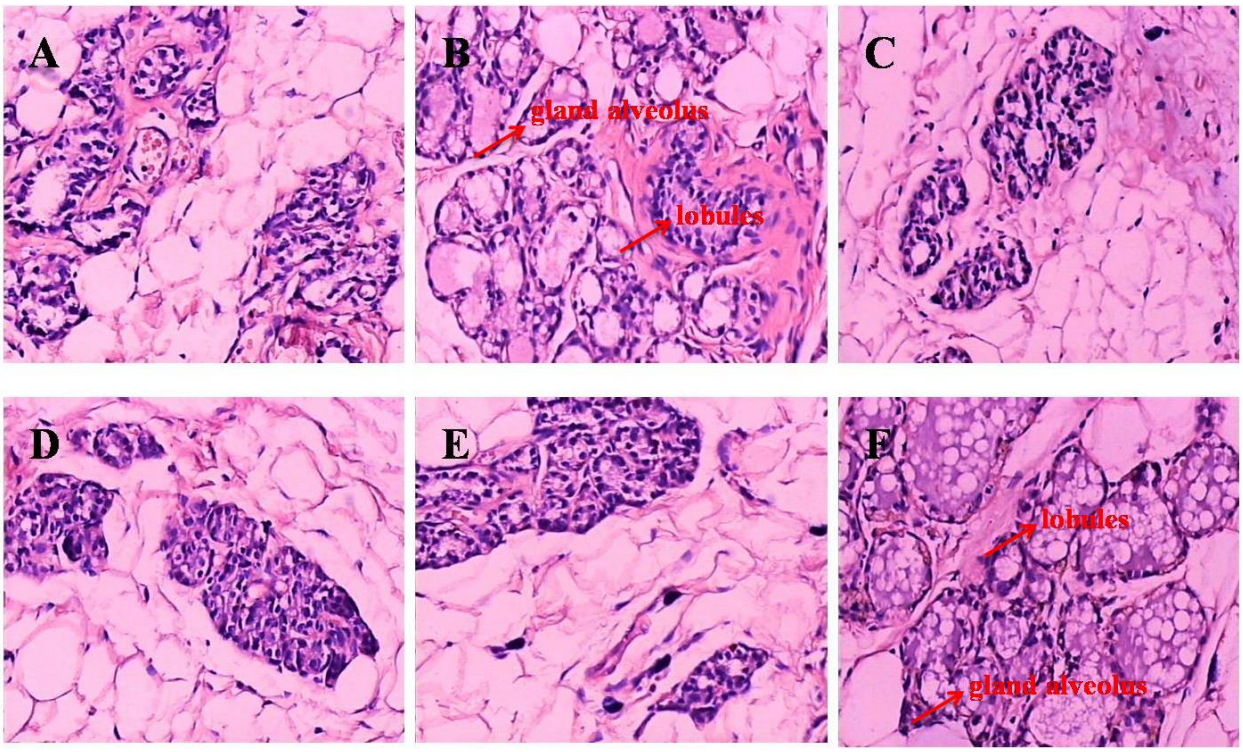

Fig. 10: Morphology of mammary gland tissue was observed by microscope (original magnification, 200×). (A) blank controls; (B) model group; (C) bromocriptine group; (D) high-dose AFH; (E) middle-dose AFH; (F) low-dose AFH.

In our experiment, the PRL concentration was determined in the hyperprolactinemia rat serum, and we found the PRL was much more than control rats. We suppose that PRL plays a very important role in the formation of HMG, and hyperprolactinemia had the potential of promoting the formation of HMG. AFH regulated PRL, E_2, P, FSH and LH effectively, thereby resumed the function of ovary and eliminated the hyperplasia of lobules and gland alveolus. We found PRL was significantly decreased and E_2, P, FSH and LH were increased by high-dose AFH treatment compared with hyperprolactinemia model group. PRL was secreted by pituitary. AFH could decreased the pituitary prolactin positive cell number and mRNA expression level of model rats significantly. Histopathologic examination of high-dose AFH treatment remarkedly alleviated the degree of HMG, number of lobules, number of acinars and lobule volumes decreased in different degrees, thus treated them effectively.

There are flavonoids, saccharides, amylasem, alkaloid, protein, catalyticase, cytochrome and candicine in *F. H. Germinatus* (Lin Jun-hong 2005; Zhang Yan-kun 2004). This work confirms the utility of the traditional uses of *F. H. Germinatus* extracts. Its total alkaloids were confirmed to have the curative effect in treating hyperprolactinemia. However, the chemical constituent of total alkaloids of *F. H. Germinatus* needs to be further identified and quantitavely analysed. In conclusion, the results presented AFH has significant therapeutic effects of treating hyperprolactinemia. It could regulate serum E2, P, PRL, FSH, LH levels, decrease the pituitary prolactin positive cell number, mRNA expression and inhibite hyperplasia of mammary gland in hyperprolactinemia model rats effectively. The total alkaloids of *F. H. Germinatus* should be developed as an anti-hyperprolactinemia product deeply.

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