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Therapeutic Effect of *Cuirutang* on Maternal Rats with Hypogalactia Induced by Bromocriptine

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ABSTRACT
To study the galactagogue effect of *Cuirutang* (a new traditional Chinese lactogenic decoction) on rats with postpartum hypogalactia, maternal rats were fed bromocriptine to induce hypogalactia and were subsequently treated with *Cuirutang*. The galactagogue effects of *Cuirutang* were evaluated based on changes in milk production, the prolactin level and the amount of prolactin receptor in the mammary gland as well as based on mass gains in the pups and mammary glands of maternal rats. The results demonstrated that *Cuirutang* significantly increased milk production ($p<0.01$), prolactin levels ($p<0.05$) and mammary gland parameters ($p<0.05$) during puerperal hypogalactia. The mass gain of the suckling pups was also significantly increased ($p<0.05$). In addition, *Cuirutang* markedly diminished the postpartum hypogalactia and mastatrophy effects induced by bromocriptine. These results demonstrate that *Cuirutang* was capable of improving the lactation function of maternal rats.

Key words: Hypogalactia, bromocriptine, mammary gland, galactagogue

INTRODUCTION

Postpartum hypogalactia, also termed “no secretion of milk” or “Insufficient milk secretion”, is an illness commonly encountered in female animals during the lactation period (Turner, 2007; Reiner *et al*., 2009). Female animals with hypogalactia exhibit little or even no milk secretion. Thus, the demand of the pups cannot be satisfied (Odegaard and Framstad, 2002). Therefore, prevention and treatment of postpartum hypogalactia is of vital significance to ensure increased pup survival and growth rates.

To date, no effective Western medical methods are available for treatment of this condition (Ilyenko *et al*., 2010). Various galactagogue agents have been developed; however, their clinical outcomes are limited (Pradhan *et al*., 2007). According to modern studies, Traditional Chinese Medicine (TCM) can achieve good results in the treatment of postpartum hypogalactia (Selukar *et al*., 2001).

According to TCM theory, postpartum hypogalactia is caused by postpartum body weakness, by qi-blood sufficiency, or by milk blockage due to Gan-qi stagnancy and other factors, such as mental tension and improper lactation methods. This statement informs other studies on postpartum hypogalactia. Treatments should nourish the qi and blood and unblock the Conception Meridian.

Cuirutang, which is composed of deficiency-nourishing herbs (*Astragalus membranaceus*, *Radix codonopsis*, *Radix angelicae sinensis*, *Rehmannia glutinosa* and *Rhizoma atractylodis macrocephalae*) and stagnancy-dredging herbs (*Medulla tetrapanacis* and *Radix rhapontici*), was formulated by following the prescription principles found in classical Chinese medical theory. By following the methods described in previous studies (Song *et al*., 2011), *Cuirutang* can be used to regulate qi and blood and dredge the collateral channel to promote lactation. The current study aimed to determine the clinical efficacy of *Cuirutang* for the prevention and treatment of hypogalactia induced by bromocriptine in rats. This study provides clinical evidence of the beneficial effects of *Cuirutang*. However, further research to determine the underlying mechanisms is warranted.

MATERIALS AND METHODS

Preparation of *Cuirutang*: All Chinese medicinal materials were purchased from the Beijing Tongrentang Pharmacy, China.

Cuirutang was prepared following the TCM method. The preparation was composed of *Astragalus membranaceus* (25 g), *Radix codonopsis* (15 g), *Radix angelicae sinensis* (10 g), *Rehmannia glutinosa* (20 g), *Rhizoma atractylodis Macrocephalae* (10 g), *Medulla tetrapanacis* (5 g), *Radix rhapontici* (5 g) and *Glycyrrhiza* (5 g). The herbs above were immersed in 1000 mL of distilled water for 30 min at room temperature and were boiled for 30 min on low heat after boiling on high heat. After filtration through 12 layers of gauze, the medicinal materials were boiled again for 20 min on low heat in 800 mL of distilled water after boiling on high heat without soaking. The decoction was then filtered with gauze again. Two decoctions were mixed and filtered with double-layered filter paper in a pressure filter. Then, the decoction was concentrated to 100 mL via vacuum concentration at 40°C and subsequently stored at 4°C.

Animals: The study was approved by the local ethics committee of the College of Veterinary Medicine, China Agricultural University. The animals were supplied by the Experimental Animal Center of the Military Medical Science Academy of the PLA (Beijing, China, license No. SCXK (jun) 2012-0004). The study protocols complied with the animal care guidelines of the National Institutes of Health (NIH).

A total of 21 healthy, female, Specific Pathogen-Free (SPF) Sprague-Dawley rats with a postpartum weight ranging from 250-270 g were used in this study. All rats were naturally mated. The rats were acclimatized to standard rat housing conditions for 21 days prior to the experiments; they were housed in plastic cages with ambient humidity on an ambient light cycle at a room temperature of 22-25°C. The rats had free access to tap water and commercial rat chow (Beijing Keaoxieli Feed Co., LTD, China).

Establishment of experimental hypogalactia: Ten postpartum female rats were randomly divided into two equal groups of 5 postpartum rats. Each mother rat was introduced to 12 young rats for breastfeeding. Group I (model group) was administered bromocriptine (Gedeon Richer Plc., Budapest, Hungary) dissolved in distilled water to 0.4 mg mL^{-1} at a dosage of 1.6 mg kg^{-1} BW. Group II (normal group) received distilled water (equivalent to the volume administered to the model group). Each rat was gavaged twice per day for 7 days (Song *et al.*, 2011). Before the first gavage on the first experimental day, the young rats with same mother in both groups were separately weighed on an electronic scale (500 g/0.01 g) and the total weights were obtained. After the first gavage on the 7th experimental day, the young rats with same mother in both groups were weighed on an electronic scale again and the total weights were obtained. The mean differences in the net weights of the young rats from each mother were determined using the least significant difference test (SPSS 20.0 software; SPSS, Chicago, IL, USA). A p-value <0.05 was considered significant. The results indicated that the mean difference between the two groups was significant and experimental hypogalactia was successfully modeled (Fig. 1).

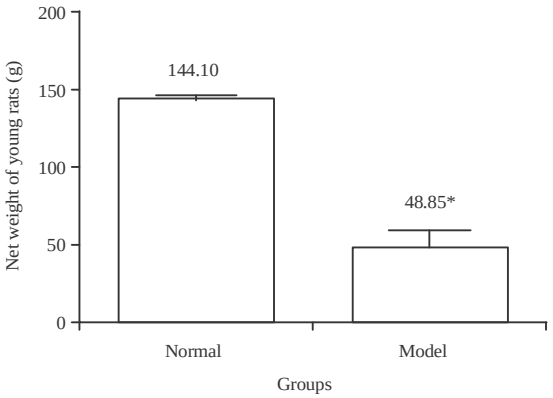

Fig. 1: Establishment of experimental hypogalactia model *p<0.05

***Cuirutang* treatment:** Twenty-one postpartum female Rats were randomly divided into 3 groups of 7 rats (Gad and El-Maddawy, 2014): The normal group, the model group and the treatment group. The treatment group was administered bromocriptine (1.6 mg kg^{-1} BW) and *Cuirutang* (4 mL kg^{-1} BW). The model group was administered distilled water (equivalent to the volume of *Cuirutang* administered to the treatment group) and bromocriptine (1.6 mg kg^{-1} BW). The normal group was administered distilled water twice (equivalent to the volumes of *Cuirutang* administered to the treatment group and bromocriptine administered to the model group). Each group was gavaged twice per day for 7 days.

After the first gavage (2 h), the total weights of the young rats with same mother in all groups were determined on the same day using an electronic scale. The weights were denoted as the pre-isolation weights. After weighing, the mothers were immediately isolated from their pups for 4 h. After the 4 h separation, the total weights of the young rats with same mother in all groups were again determined using an electronic scale. This weight was denoted as the pre-breast-feed weight. Then, the mothers were allowed to freely breastfeed their pups for 1 h. After 1 h, the total weights of the young rats with same mother in all groups were again determined using an electronic scale. This weight was denoted as the post-breastfeed weight. After the third weigh-in, the mothers freely breastfeed their pups. The weighing test was repeated for 7 days.

After the last weigh-in on the 7th day, all mothers were weighed on an electronic scale and euthanized at the end of the experiment. Blood samples were collected from the inferior vena cava. After animal dissection, the mammary glands was grossly examined, photographed, rapidly removed and weighed. Equivalent portions of each mammary gland were

excised and placed in 4% neutral buffered paraformaldehyde for histopathological analysis. The remaining samples were frozen in liquid nitrogen and stored at -80°C for subsequent assessments.

Lactation quantity per hour: According to Noblet's Method (Noblet and Etienne, 1989), the pup weight gain could be used as an estimate of milk yield. The lactation quantity per hour was calculated using the following formula W3-W2+(W1- W2)/4.

W3: post-breastfeed weight; W2: pre-breastfeed weight; W1: pre-isolation weight.

Net weights of the young rats: The net weights of the young rats were obtained by determining the difference between the pre-isolation weight on the 1st day and the post-breastfeed weight on the 7th day.

Mammary gland parameters: The index weight (I.W.) of each mammary gland was calculated according to Matousek (1969), where I.W. = organ weight (g)/body weight (g) * 100.

Prolactin (PRL) serum levels and mammary gland tissue homogenates: The blood samples were clotted for 2 h at room temperature and centrifuged for 15 min at 1000×g. The serum was aliquoted and stored at -80°C. A 100 mg tissue sample was rinsed with 1X PBS, homogenized in 1 mL of 1X PBS and stored overnight at -80°C. After two freeze-thaw cycles, the samples were centrifuged at 5,000×g at 4°C for 5 min. The supernatant was removed, aliquoted and stored at -80°C. The serum and tissue homogenates were analyzed using High-Range Rat Prolactin ELISA kits (Mouse #; CSB-e06882 m, CUSABIO BIOTECH CO., Ltd., China).

Mammary gland structure: The paraformaldehyde-treated mammary gland tissues were dehydrated and embedded in paraffin. Paraffin sections (5 μm thickness) were prepared and stained with hematoxylin and eosin (HE). Then, pathological alterations were observed and photographed under a microscope (Nikon Digital Sight ds-ril, Japan) (Bancroft and Cawood, 1996).

Western blot assay for the PRL receptor: Phenylmethyl sulfonylfluoride (100 mM L^{-1}) was added to the protein extraction reagent. The frozen mammary glands were lysed in protein extraction reagent (1:9), homogenized on ice and centrifuged at 10,000 rpm for 20 min. The supernatant was stored at -80°C. The protein concentrations were measured using the bicinchoninic acid assay. Protein samples were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel for 2 h and transferred onto a membrane for 1 h. The membranes were blocked by incubation in 5% skimmed milk powder with rotation for 1 h; subsequently, they were incubated with primary antibodies, including rabbit anti-rat PRL receptor protein (1:20,000; Epitomics, Burlingame, CA, USA) and rabbit anti-rat β-actin (1:1,000; Abcam, Cambridge, UK), at 4°C for 20 h. The samples were then incubated in goat anti-rabbit secondary antibody (IgG, 1:20,000; ANR02-1, Beijing, China) for 1 h at room temperature. The membranes were washed four times for 5 min each wash, incubated in enhanced chemiluminescence reagent for 5 min, exposed with Kodak film (Comwin, Beijing, China) in a darkroom, visualized and fixed. After air-drying, the X-ray film was scanned using an YLN-2000 gel image analysis system (Yalien, Beijing, China).

ImageJ software (NIH, Santa Clara, CA, USA) was used to process the images and Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA) was used to read the integrated absorbance values. The relative amounts of target proteins were calculated by determining the integrated absorbance ratio of target protein/internal reference (β-actin).

Statistical analysis: The data were statistically analyzed using SPSS 20.0 software (SPSS, Chicago, IL, USA). The mean differences among the groups were compared using one-way analysis of variance followed by Duncan's multiple range test. Paired comparisons of intergroup data were performed using the least significant difference test. The p-values <0.05 were considered significant.

RESULTS

Lactation yield per hour: After bromocriptine induction, a significant decrease in lactation was observed in the model group compared with the normal group on days 3-7 ($p<0.01$). The lactation yield per hour of the model group was decreased compared with that of the normal group on the 3rd day ($p<0.05$) and it was decreased significantly compared with that of the normal group on days 4-7 ($p<0.01$). Both the normal group and the treatment group demonstrated obvious increases in lactation yields per hour during the 7 days and the lactation yield in the treatment group was not significantly lower than that in the normal group on any given day (Fig. 2).

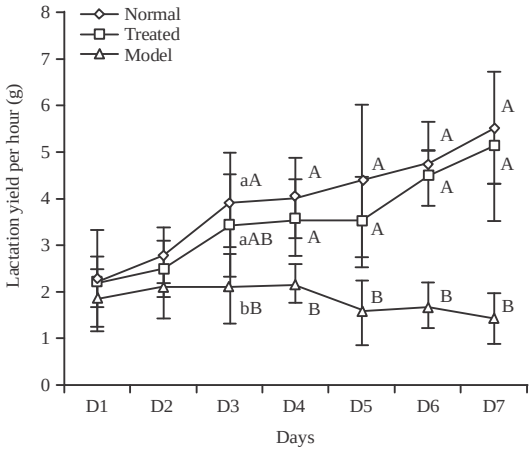

Fig. 2: Lactation yield per hour. Different letters indicate significant differences (lower case indicates $p<0.05$, upper case indicates $p<0.01$)

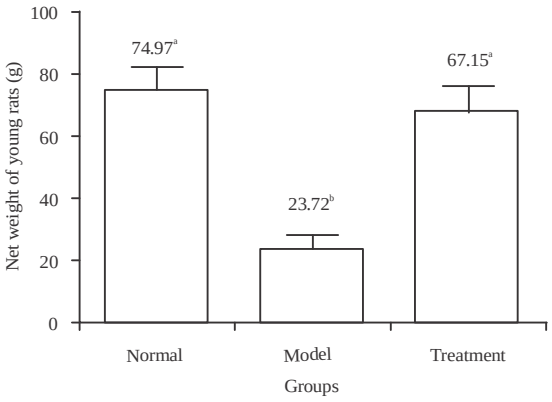

Fig. 3: Net weights of young rats. Different superscripted letters indicate significant differences

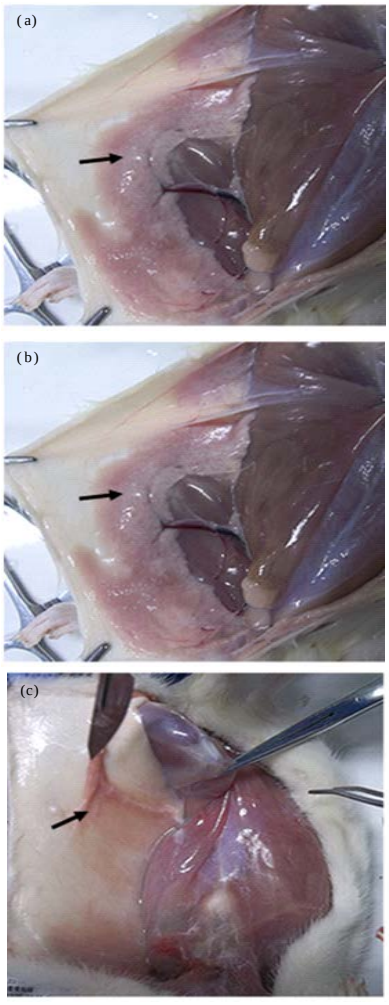

Fig. 4(a-c): Comparison of mammary glands, (a) Normal, (b) Treatment and (c) Model group

The treatment group was supplemented with *Cuirutang* at the prescribed dose and demonstrated significant increases in lactation yields per hour compared with the model group

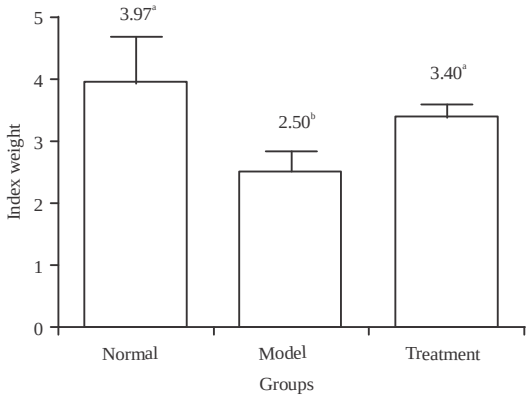

Fig. 5: Mammary gland parameters. Different superscripted letters indicate significant differences

($p<0.01$). This result suggests that *Cuirutang* effectively increased the lactation yield in hypogalactic rats (Fig. 2).

Net weights of young rats: After 7 days of treatment, there was no significant difference in the net weights of young rats in the treatment group and the normal group. The increase in net weight was significantly higher in the treatment group compared with the model group ($p<0.05$), suggesting that *Cuirutang* increased the secretion volume and net weight of young rats in the treatment group (Fig. 3).

Comparison of mammary glands: After 7 days of treatment, the mammary glands of rats in the model group did not appear to be well distributed in the subcutaneous thoraco-abdominal area compared with those in the normal group. Additionally, the mammary glands were dull and pale yellow in appearance (Fig. 4a-c). In the treatment group, the distribution area and volume of the mammary glands were recovered to the same levels observed in the normal group. The mammary glands appeared shiny and reddish-brown, suggesting that *Cuirutang* restored the distribution area and volume to their normal levels (Fig. 4a-b). There was no significant difference in the I.W. of the mammary gland between the treatment and normal groups. The mammary gland I.W. after 7 days of *Cuirutang* treatment in the treatment group was significantly increased in the treatment group compared with the model group ($p<0.05$) (Fig. 5), suggesting that *Cuirutang* increased rat mammary gland proliferation in the treatment group.

Serum and tissue PRL levels: On the 7th day, there were no significant differences in the serum PRL levels or tissue PRL levels of mammary glands in the treatment and normal groups. However, the serum PRL level of the treatment group was higher than that of the normal group (Fig. 6a); this result was the opposite of that observed for the tissue PRL level in the mammary glands (Fig. 6b). Compared with the model group, both the serum PRL level and the tissue PRL level of the mammary glands in the treatment group were significantly increased on the 7th day after *Cuirutang* treatment ($p<0.05$), suggesting that *Cuirutang* increased the PRL level in the treatment group (Fig. 6a-b).

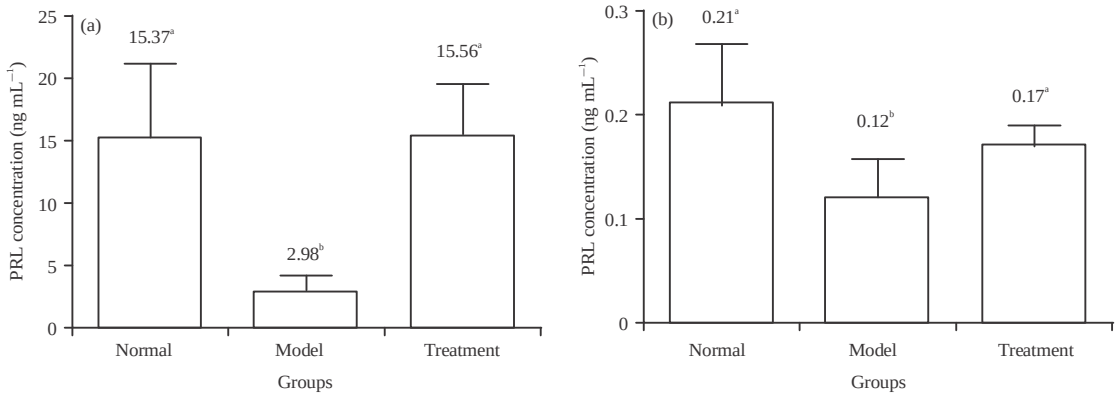

Fig. 6(a-b): PRL concentration, (a) Serum PRL concentration and (b) PRL concentration in mammary gland homogenates. Different superscripted letters indicate significant differences

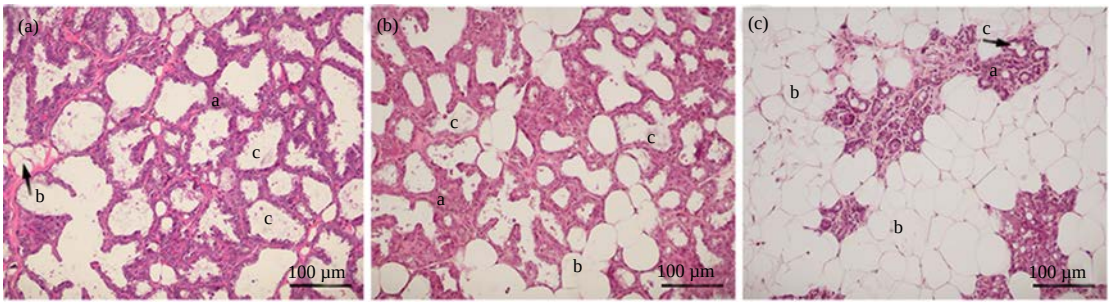

Fig. 7(a-c): Mammary gland structure. HE staining, scale bars: 100 μm, (a) Normal group, (b) Treatment group and (c) Model group, a: Mammary gland lobule, b: Adipose cell and c: Acinus

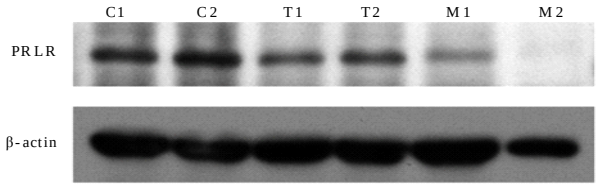

Fig. 8: PRL receptor protein expression in mammary gland C1, C2: Normal, T1, T2: Treatment and M1, M2: Model group

Mammary gland structure: Compared with the normal group, rats in the model group showed significant mammary gland atrophy. The levels of adipose and connective tissues were significantly increased in the mammary glands and mammary gland lobules were not obvious. The acinar cavities were decreased significantly; acini were sparse and scattered and only individual cavities contained small amounts of secretion. After 7 days of treatment, the typical organization of the mammary gland was restored. The amounts of adipose tissue and connective tissue were significantly decreased in the mammary glands of rats in the treatment group compared with those in the model group. The number of acini and mammary gland lobules increased significantly and large amounts of secretion were visible in a many acini, suggesting that *Cuirutang* restored the normal structure of the mammary gland (Fig. 7).

Comparative analysis of the PRL receptor: Western blot assays demonstrated that compared with the normal and treatment groups, expression of the PRL receptor protein was significantly lower in the model group on the 7th day ($p<0.05$). Although, the expression of the PRL receptor protein in the treatment group was also lower than that in the normal group, this difference was not significant (Fig. 8).

DISCUSSION

From the perspective of modern medicine, lactogenesis is a process in which udder epithelial cells change from non-secretory cells to secretory cells; thereafter, they secrete milk into acini (Tucker, 1979). It is known that PRL plays an essential role in milk synthesis and secretion during lactation and it is needed for the onset of lactation and maintenance. Moreover, the PRL concentration is also positively correlated with mammary gland wet weight (Farmer, 2001). Bromocriptine mesylate is a semisynthetic ergot alkaloid derivative with potent dopaminergic activity. It inhibits

PRL secretion and decreases plasma PRL levels in rats (Ribeiro-De-Oliveira *et al.*, 2001). The observed effect of bromocriptine on PRL concentrations was associated with changes in PRL levels in our study. Additionally, the lactation yield, rat pup growth and mammary gland results in our present study agree with previous findings.

Due to the possibility that allopathic drugs may cause allergic reactions and negative side effects, attempts are being made to revert to herbal treatments all over the world. An increasing number of effective TCM herbs with physiological effects on the structure and function of different body tissues are being identified and used for the treatment and prevention of diseases (Williamson *et al.*, 2013). The efficacy of preventive applications of herbal preparations for hypogalactia syndrome has been previously reported (Jayashree *et al.*, 1999; Gajecki *et al.*, 2001). Additionally, herbal therapies have been shown to have remedial effects on hypogalactia (Selukar *et al.*, 2001; Mishra and Agrawala, 2002; Di Pierro *et al.*, 2008). In our study, the use of *Cuirutang* in rats led to considerable success in the treatment of hypogalactia.

To understand the subtleties of this method, we examined the prescription to preliminarily analyze the herbal effects. All prescription herbs were first documented in Shennong Bencao Jing (Shennong's Classic of Materia Medica, 200- 300 AD) and have been used as medicines for more than 2000 years. Today, these herbs are still the most commonly used herbs by TCM practitioners in China and Europe (Williamson *et al.*, 2013). In accordance with the classification of TCM, *Astragalus membranaceus* (known as Huangqi in Chinese), *Radix codonopsis* (known as Dangshen in Chinese), *Radix angelicae sinensis* (known as Danggui in Chinese), *Rehmannia glutinosa* (known as Dihuang in Chinese) and *Rhizoma atractylodis macrocephalae* (known as Baizhu in Chinese) are deficiency-nourishing herbs, which are the most popular health-promoting herbal medicines commonly used for benefiting qi, nourishing blood and promoting blood circulation. *Medulla tetrapanacis* (known as Tongcao in Chinese) and *Radix rhapontici* (known as Loulu in Chinese) are stagnancy-dredging herbs, which are commonly used for dredging collateral channels to promote lactation. *Glycyrrhiza* (known as Gancao in Chinese) is used as an adjuvant in prescriptions, which mitigates the fierce action of primary medicines and protects the gastric qi according to the "treatise on febrile diseases". Experience in medical practice has verified that the reasonable selection of drugs is essential for improving the clinical therapeutic effects (Fuhr, 2000). There is no negative evidence concerning the combination of deficiency-nourishing herbs and stagnancy-dredging herbs with the adjuvant described above for hypogalactia treatment in China. Modern phytochemistry and pharmacological experiments have demonstrated that prescription herbs and their active components have various important bioactivities (Hook, 2014; Jin *et al.*, 2014) and exhibit broad pharmacological functions throughout the blood system, immune system, endocrine system, cardiovascular system and nervous system (Zhang *et al.*, 2008; Yan *et al.*, 2013).

Therefore, because the components are complicated, the mechanistic action of *Cuirutang* deserves further study, which may provide targets for novel drug development and clinical applications.

CONCLUSION

In summary, *Cuirutang* significantly increased milk production, PRL levels and mammary gland parameters in maternal rats with puerperal hypogalactia. *Cuirutang* also improved the mass gain of suckling rat pups and markedly diminished the postpartum hypogalactia and mastatrophy induced by bromocriptine. In addition, there were no significant differences in any of the parameters above between the treatment and normal groups. This study demonstrated that *Cuirutang* improved the lactation function of maternal rats with hypogalactia and can therefore be used to treat postpartum hypogalactia induced by bromocriptine. Thus, *Cuirutang* should be clinically applied.

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