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Original Research Article

Anti-Hyperprolactinemic Effect of Formula Malt Decoction, a Chinese Herbal Cocktail

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Abstract

Purpose: *To investigate the anti-hyperprolactinemic activity of Formula Malt Decoction (FMD), a Chinese herbal mixture.*
Methods: *The effect of FMD on serum prolactin (PRL), estradiol (E2), progesterone (PGN), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were investigated in hyperprolactinemic (hyperPRL) rats. The effect of FMD on PRL secretion, dopamine D2 receptor and dopamine transporters (DAT) were studied in MMQ cells derived from rat pituitary adenoma cells, GH3 cells derived from rat pituitary lactotropic tumoral cells and PC12 cells from rat pheochromocytoma, respectively.*
Results: *Compared with the model group, a high dose of FMD (11.6 g/kg body weight) and medium dose of FMD (5.8 g/kg body weight) reduced PRL level of hyperPRL rats effectively. In MMQ cells, treatment with 5 mg/ml FMD ($p < 0.01$) or 10 mg/mL FMD ($p < 0.01$) significantly suppressed PRL secretion and synthesis at 24 h compared with controls ($p < 0.01$). Consistent with D2- action, FMD did not affect PRL in rat pituitary lactotropic tumor-derived GH3 cells that lack the D2 receptor expression compared with controls, but 8 mg/mL FMD significantly increased the expression of D2 receptors ($p < 0.01$) and DAT ($p < 0.01$) in PC12 cells.*
Conclusion: *FMD shows anti-hyperprolactinemia activity via dopamine D2 receptor.*

Keywords: *Formula Malt Decoction, Hyperprolactinemia, Dopamine D2 Receptor, Prolactin, Estradiol, Progesterone follicle stimulating hormone, Luteinizing hormone*

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INTRODUCTION

Hyperprolactinemia, is one of the most common endocrine disorders of the hypothalamus-pituitary axis (PRL > 25 ng/mL) in young women. It is associated with galactorrhea and ovulatory dysfunction that result in menstrual irregularities and bareness [1]. HyperPRL 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 and polycystic ovarian syndrome [2,3]. Synthetic drugs are used in treating them, but they always bring many side effects such as menstrual disorder and the relapse rate is very high. It has been reported that 12 % patients cannot endure bromocriptine, which is a widely used synthetic drug in clinical practice [4].

Empirical evidence suggests that many herbal medicines possess therapeutic potential for alleviating hyperPRL symptoms [5]. Formula Malt

Decoction is widely used in China for the treatment of hyperprolactinemia and has produced a favorable effect. Formula Malt Decoction (FMD) is composed of 60 g Mai Ya (*Fructus Hordei Germinatus*), Xia Ku Cao (*Prunella vulgaris* L.) 15 g, Chai Hu (*Bupleurum chinense* DC.), Dang Gui (*Angelica sinensis*), and 10 g Dan Shen (*Salvia miltiorrhiza* Bge.). 10 g *Fructus Hordei Germinatus*, the main herb of FMD, and its extract decreases prolactin levels in hyperPRL mice [6,7].

Most conventional anti-hyper PRL agents reduce PRL secretion through D2 receptor in the hypothalamic-neuroendocrine dopaminergic system [8]. Other sex steroids are also involved in the pathophysiology of hyperPRL [9]. In this study, we investigated the effects of FMD on serum E2, P, FSH and LH levels in hyper PRL rats; as well as its effect on D2 receptor- and DAT-mediated responses and PRL secretion in cell- culture systems. MMQ cell derived from rat pituitary adenoma cells, GH3 cell derived from rat pituitary lactotropic tumoral cells and PC12 cells from rat pheochromocytoma were used.

EXPERIMENTAL

Preparation of FMD

The dried and crushed herbs of FMD were mixed, and decocted with 1100 ml water twice, 45 min for each. Then the filterate was mixed and concentrated into 200 ml liquor of FMD. After being filtered through a 0.2 μm filter, the liquor of FMD was used for *in vitro* and *in vivo* experiments.

In vivo experiments in animal model of hyperPRL

Female Wistar rats weighing 200 - 220 g were obtained from Shandong Center for Disease Control and Prevention, Jinan, Shandong. The animals had free access to feed 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 of Shandong University of Traditional Chinese Medicine (Approval reference NO. 20130912) and was carried out in compliance with the Directive 2010/63/EU on the handling of animals used for scientific purposes [10].

Rats were given intraperitoneal (i.p.) metoclopramide (MCP, 150 mg/kg body weight daily) for 10 days to prepare the model of hyper PRL [11]. 60 rats were randomly divided into six groups of ten rats: control, model group, bromocriptine-treated group, high-dose FMD-treated (11.6 g/kg body weight) group, middle-dose FMD-treated (5.8 g/kg body weight) group and low-dose FMD-treated (2.9 g/kg body weight) group. Each dose was dissolved in 2 ml water, and administered by gavage. The dosage was calculated from the human clinical dosage of FDM based on body surface area. Control and untreated model rats received 2 mL of saline water. All the mice received treatment for 30 days. After finishing the treatment serum PRL, E2, PGN, FSH and LH levels of the rats were measured by ELISA.

In vitro experiments in culture cells

Cell lines and culture

MMQ cell which is derived from rat pituitary adenoma cells, GH3 cell (derived from rat pituitary lactotropic tumoral cells) and PC12 cell (derived from rat pituitary lactotropic tumor) were used in the in vitro experiments. Dose-dependent and time-course responses of PRL secretion and synthesis to FMD treatment were evaluated in MMQ cell line. Effects of FMD on dopamine D2 receptor and DAT were further examined in PC12 cells. MMQ, GH3 and PC12 cell lines were cultured in 65 cm^2 flask, supplemented with 5 % fetal calf serum (FBS), 11 % heat-inactivated horse serum (HS), penicillin (100 IU/ml), and streptomycin (100 μg/mL) under a humidified atmosphere containing 5 % CO_2 at 37 °C. The culture medium was replaced with fresh medium every two days. When the density reached 75 % confluence, culture cells were transferred to 35 mm-diameter 6-well plates for experimental treatment.

MMQ cells were treated with FMD at concentrations of 1-8 mg/mL for 12–48 h. At different incubation time points, the culture medium was collected for measuring PRL secretion. Cells were collected for determining cellular PRL expression. The optimal concentrations and treatment duration were then determined for subsequent experiments. GH3 and PC12 cells were treated with FMD at effective concentrations that had been determined in MMQ cells for 24 h. The GH3 cells and medium were collected for the measurement of PRL secretion and expression respectively. The PC12 cells were collected for detecting D2 receptor and DAT expressions.

Biochemical analysis

Hormone assays

PRL concentrations in the culture medium collected from MMQ and GH3 cells as well as

serum PRL, E2, PGN, FSH and LH concentrations of rat were measured using ELISA kits (Wuhan boster Biological Engineering Co, Ltd).

Western blotting

Expressions of intracellular PRL in MMQ and GH3 cells as well as D2 receptors and DAT in PC12 cells were determined by western blotting. The cell proteins were extracted and the concentration was determined by Bradford method. Proteins were separated by a 10 % SDS-PAGE gel and transferred electrophoretic ally onto nitrocellulose membranes (Bio Basic, Inc). Immune detection was performed with the primary antibodies against PRL, D2 receptors, and DAT at a dilution of 1:1000 at 4 °C overnight, followed by co-incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 30 min at room temperature. The primary antibody was served against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a standard control for protein loading. The chemiluminescence was determined by ECL detection kits (Shang hai Best Bio, China). The intensity of protein bands was quantified by scanning densitometry using Quantity One 4.5.0 software.

Statistical analysis

One- or two-way analysis of variance (ANOVA) was used to detect statistical significance, followed by post-hoc multiple comparisons (Student-Newman-Keuls method). Data are expressed as mean ± SEM. Statistical significance was defined as $p < 0.05$.

RESULTS

Effect of FMD on sex hormones in rat model of hyperPRL

Compared with control group, serum PRL level of hyperPRL model rats increased significantly ($p < 0.01$). Serum E2, PGN and FSH and LH ($p < 0.05$) levels of hyperPRL model rats decreased significantly ($p < 0.01$) after treatment with intraperitoneal metoclopramide.

The increased PRL level was significantly attenuated by treatment with 0.6 mg/kg bromocriptine and 5.8 or 11.6 g/kg FMD after 30 days ($p < 0.01$) compared with model group. Compared with model group, rat serum E2 level decreased ($p < 0.01$), and serum PGN, FSH and LH levels increased ($p < 0.05$) effectively in bromocriptine-treated group. Compared with model group, rat serum E2 level decreased ($p < 0.01$), and serum P, FSH and LH levels increased ($p < 0.05$) significantly treated by high-dose FMD (Table 1).

Suppression of hyperactive PRL in MMQ and GH3 cells by FMD

Two-way ANOVA analysis revealed a significant interaction between time course and treatment dose ($F = 2.226$, $p = 0.048$). Post-hoc multiple comparison further revealed that 1 and 10 mg/mL FMD treatment for 24 and 36 h, but not 12 and 48 h, produced significant suppression of PRL concentrations in the MMQ culture medium compared with controls (0 mg/mL) ($p < 0.006$) (Fig. 1A). 24 h were then chosen.

FMD treatment for 24 h also yielded a significant suppression of MMQ cellular PRL expression in a dose-dependent manner ($F = 28.135$, $p < 0.001$). Compared to controls, the significant suppression was observed in the higher two concentrations (5 and 10 mg/mL) ($p < 0.003$) (Fig. 1B). The same concentrations did not produce the significant effects on the medium concentration ($F = 2.535$, $p = 0.115$) and the cellular expression ($F = 0.267$, $p = 0.862$) in GH3 cells (Fig. 2).

Table 1: Effect of FMD on sex hormones in hyperPRL rats

Group	PRL (pg/mL)	E2 (pmol/L)	P (ng/mL)	FSH (IU/L)	LH (mIU/mL)
Control	218.54 ± 6.52	3.59 ± 0.32	1.21 ± 0.05	0.97 ± 0.06	1.81 ± 0.13
Model	432.14±35.57**	1.36 ± 0.18**	0.55 ± 0.15**	0.36 ± 0.04**	0.97 ± 0.06*
Bromocriptine	225.69±11.70$^{\triangle}$	2.56 ± 0.26$^{\triangle\triangle}$	0.73 ± 0.03$^{\triangle}$	0.71 ± 0.02$^{\triangle}$	1.62 ± 0.05$^{\triangle}$
FMD-H	226.15±11.51$^{\triangle}$	2.55 ± 0.15$^{\triangle\triangle}$	0.86 ± 0.05$^{\triangle}$	0.62 ± 0.02$^{\triangle}$	1.71 ± 0.21$^{\triangle}$
FMD-M	251.62±14.63$^{\triangle}$	2.34 ± 0.38$^{\triangle}$	0.81 ± 0.06$^{\triangle}$	0.67 ± 0.03$^{\triangle}$	1.58± 0.04$^{\triangle}$
FMD-L	320.26 ± 47.22	1.86 ± 0.46	0.54 ± 0.11	0.53 ± 0.07	1.02 ± 0.12

*Data are expressed as mean ± SEM (n =10) and analyzed using one-way ANOVA. *p < 0.05 and **p < 0.01 versus control group; $^{\triangle}p < 0.05$ and $^{\triangle\triangle}p < 0.01$ versus model group.* ***Key:*** *FMD-H = high-dose FMD; FMD-M = medium-dose FMD; and FMD-L = low-dose FMD*

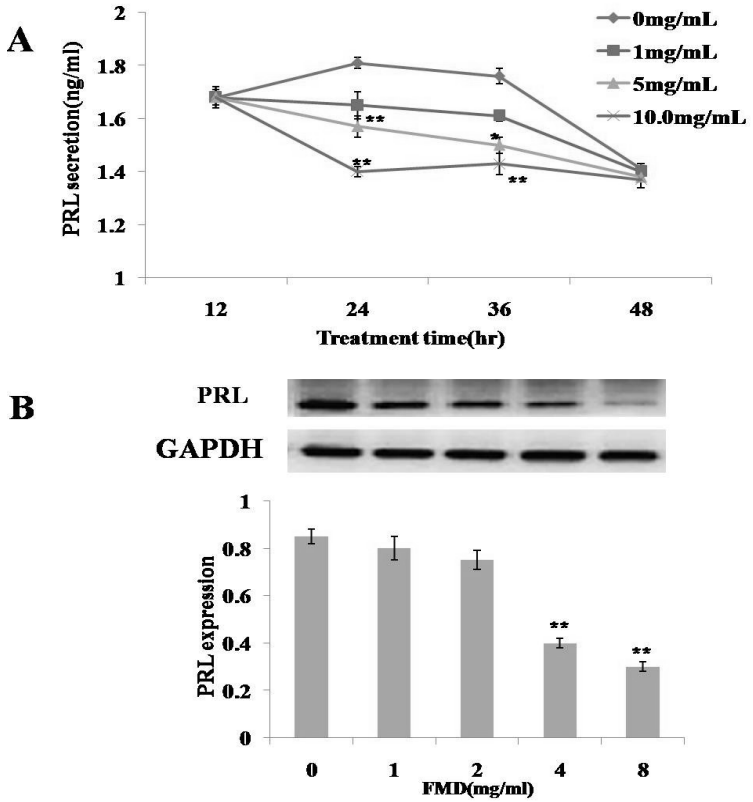

Fig 1: Time-course and dose-dependent effect of FMD in suppressing PRL secretion (A), and dose-dependent effect of FMD in inhibiting intracellular PRL expression (B) in MMQ cells. For the intracellular PRL expression, the cells were treated with different concentrations of FMD for 24 h. Data are expressed as mean ± SEM (n = 3) and analyzed using one- or two-way ANOVA; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus 0 mg/mL group

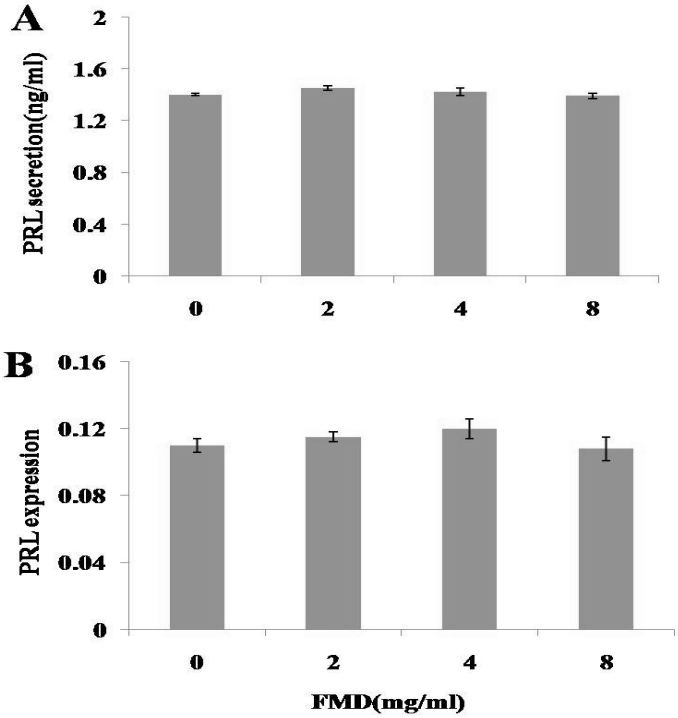

Fig 2: Effect of 24 h FMD treatment on PRL secretion (A) and synthesis (B) in GH3 cells. Data are expressed as mean ± SEM (n = 3) and analyzed using one-way ANOVA. No significant differences were found in any multiple comparisons

Enhancement of dopamine expression in PC12 cells by FMD

PC12 cells were treated with 2-8 mg/mL concentrations of FMD for 24 h. One-way ANOVA revealed that FMD increased the expression of D2 receptors significantly ($F = 6.147$, $p = 0.004$) and DAT ($F = 5.715$, $p = 0.013$) (Fig. 3). Multiple comparisons further exhibited that significant effects were attributed to concentrations of 4 mg/ml and 8 mg/ml compared to controls for D2 receptors ($p < 0.013$) and DAT ($p < 0.029$).

DISCUSSION

MMQ cells, which express D2 receptor, are an exemplary model of hyperPRL derived from rat pituitary adenoma cells responsive to dopamine [12]. Dose-dependent and time-course responses of PRL secretion and synthesis to FMD treatment were evaluated in this cell line. GH3 cells were derived from rat pituitary lactotropic tumoral cells that lack D2 receptor expression [13,14], which were used to determine if deficiency of D2 receptors altered the suppression of FMD on hyperactive PRL. Consistent with D2- action, FMD did not affect PRL in rat pituitary lactotropic tumor-derived GH3 cells that lack the D2 receptor expression. These studies revealed D2 receptor is necessary for anti-hyperPRL activity of FMD. PC12 cells from rat pheochromocytoma abundantly express D2 receptors and DAT [15,16]. FMD significantly increased the expression of D2 receptors and DAT in PC12 cells. Results suggested that D2 receptor and DAT all played important roles in anti-hyperPRL activity of FMD.

Dopamine receptors belong to the family of seven transmembrane domain G-protein coupled receptors (GPCR) [17-19]. Dopamine receptors D1 and D2 are classified into two subfamilies based on their differential effect on adenylyl cyclase.

Classically, the functions of dopamine receptors have been associated with the regulation of adenylate cyclase-protein kinase A through G-protein-mediated signaling. Two classes of GPCR mediate dopamine functions, D1-like receptor subtypes (D1 and D5) couple mostly to Ga_s and stimulate the production of the second messenger cyclic adenosine monophosphate

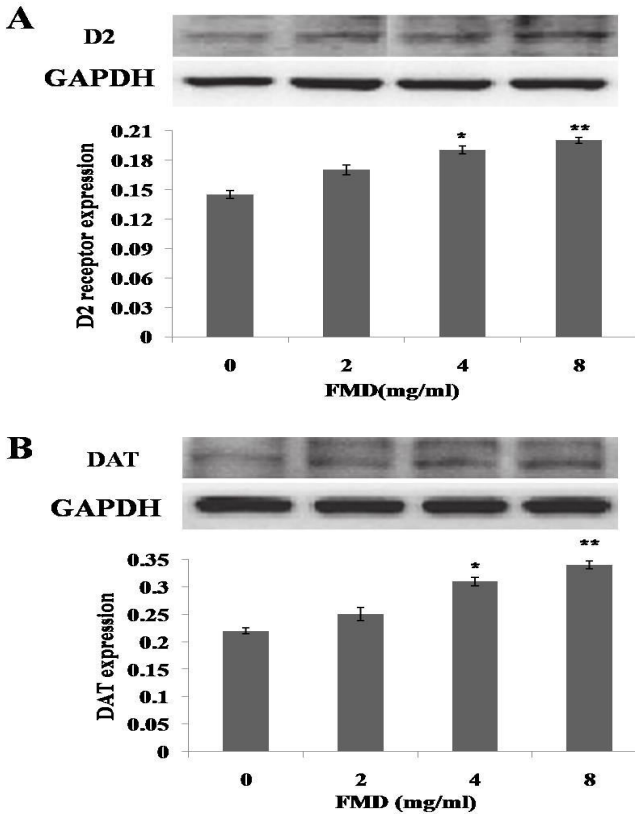

Fig 3: Effect of 24 h FMD treatment on the D2 receptor expressions (A) and DAT expressions (B). Data are expressed as mean ± SEM (n = 4) and analyzed using one-way ANOVA. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus 0 mg/ml group

(cAMP) and the activity of protein kinase A (PKA). By contrast, D2-like subfamily (D2, D3 and D4) couple to $Ga_{i/o}$ and regulate the production of cAMP thus resulting in a diminution of PKA activity [20-22]. The physiological and pathological roles of DR2 have been recognized in some organs such as brain and kidney [23,24]. In the adenohypophysis, the predominant dopamine receptor is the D2 receptor [25]. Transfection of the dopamine D2 receptor into a pituitary cell line results in a decrease in intracellular cAMP and inhibits prolactin secretion when dopamine is added to the cell culture [26]. Most conventional anti-hyperPRL agents reduce PRL secretion through D2 receptor in the hypothalamic neuroendocrine dopaminergic system. In our study, FMD inhibited PRL secretion via dopamine D2 receptor in MMQ cell. These suggest that FMD showed anti-hyperPRL activity via dopamine D2 receptor.

CONCLUSION

The findings of this study show that FMD inhibits PRL secretion in hyperPRL rats effectively. D2 receptor and DAT play important roles in the anti-hyperPRL activity of FMD.

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Ming-xia Li and Hong Liu contributed equally to this work.

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