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 lactotrophic 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 lactotrophic 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

INTRODUCTION

Hyperprolactinemia, is one of the most common endocrine disorders of the hypothalamic-pituitary axis (PRL > 25 ng/mL) in young women. It is associated with galactorrhea and ovulatory dysfunction that result in menstrual irregularities and barreness [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 filtrate 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 lactotropin tumoral cells) and PC12 cell (derived from rat pituitary lactotropin 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 cm² 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% CO₂ 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 electrophoretically 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 (Shanghai Co, Ltd). Immune detection was performed with the secondary antibodies against PRL, D2 receptors, and DAT at a dilution of 1:1000 at 4 °C overnight.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>PRL (pg/mL)</th>
<th>E2 (pmol/L)</th>
<th>P (ng/mL)</th>
<th>FSH (IU/L)</th>
<th>LH (mU/mL)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>218.54 ± 6.52</td>
<td>3.59 ± 0.32</td>
<td>1.21 ± 0.05</td>
<td>0.97 ± 0.06</td>
<td>1.81 ± 0.13</td>
</tr>
<tr>
<td>Model</td>
<td>432.14±35.57**</td>
<td>1.36 ± 0.18**</td>
<td>0.55 ± 0.15**</td>
<td>0.36 ± 0.04**</td>
<td>0.97 ± 0.06*</td>
</tr>
<tr>
<td>Bromocriptine</td>
<td>225.69±11.70Δ</td>
<td>2.56 ± 0.26ΔΔ</td>
<td>0.73 ± 0.03Δ</td>
<td>0.71 ± 0.02Δ</td>
<td>1.62 ± 0.05Δ</td>
</tr>
<tr>
<td>FMD-H</td>
<td>226.15±11.51Δα</td>
<td>2.55 ± 0.15ΔΔα</td>
<td>0.86 ± 0.05Δ</td>
<td>0.62 ± 0.02Δ</td>
<td>1.71 ± 0.21Δ</td>
</tr>
<tr>
<td>FMD-M</td>
<td>251.62±14.63Δ</td>
<td>2.34 ± 0.38Δ</td>
<td>0.81 ± 0.06Δ</td>
<td>0.67 ± 0.03Δ</td>
<td>1.58±0.04Δ</td>
</tr>
<tr>
<td>FMD-L</td>
<td>320.26±47.22</td>
<td>1.86 ± 0.46</td>
<td>0.54 ± 0.11</td>
<td>0.53 ± 0.07</td>
<td>1.02 ± 0.12</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n = 10) and analyzed using one-way ANOVA. *p < 0.05 and **p < 0.01 versus control group; αp < 0.05 and ΔΔp < 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 lactotrophic 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 lactotrophic 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 \( \text{Ga}_\alpha \) and stimulate the production of the second messenger cyclic adenosine monophosphate
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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