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Article

Traditional Mongolian medicine RuXian-1 suppress the breast hyperplasia by GRP78/PERK/CHOP pathway in rat model

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Abstract: In this study we aimed to study the protective effect of RuXian-1, which is a traditional Mongolian medicine on hyperplasia of mammary glands in rats and to explore its possible mechanism. Methods: The rat model of breast hyperplasia was induced by intramuscular injection of estradiol benzoate. The rats were randomly divided into 4 groups: saline treatment group (negative control), estrogen treatment group (model group), RuXian-1 treatment group and raloxifene treatment group (positive control). RuXian-1 group and raloxifene group were given 3.0 g/kg of RuXian-1 and 1.8 mg/kg of raloxifene daily for 4 weeks, respectively. After treatment, the breast tissue of each group was harvested and confirmed the expressions levels of glucose-related protein 78 (GRP78), C/EBP homologous protein (CHOP), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) which were known that functions as an endoplasmic reticulum stress marker by Western blotting. Results: Compared with the normal group, the GRP78, PERK and CHOP protein levels in the breast tissue of the model group were significantly decreased. Compared with the model group, the expression of GRP78, PERK and CHOP in RuXian-1 group significantly increased, the expression of GRP78 and CHOP of raloxifene significantly increased ($p < 0.05$), and the expression of PERK did not change. Conclusion: The Mongolian medicine RuXian-1 has therapeutic effect on mammary hyperplasia in rats, and its therapeutic mechanism is related to activating the GRP78/PERK/CHOP signaling pathway and promoting mammary gland cell apoptosis.

Keywords: mammary gland hyperplasia; Mongolian medicine; RuXian-1; GRP78/PERK/CHOP signaling pathway

Hyperplasia of mammary gland (HMG) is the most common and frequent breast disease in middle-aged women and is highly associated with breast cancer. HMG is a condition in which there is an excessive growth of breast tissue, resulting in enlargement of the breasts. Currently, the prevalence of breast enlargement accounts for more than 70% of all breast diseases [1]. The major signs/symptoms of HMG are as follows: clinical/non-clinical breast pain, nodular breast, or nodular breast mass. Such as bilateral or unilateral breast lumps of different sizes, lumps in the form of flakes, nodules or mixed, moving to touch, with clear margins and unchanged skin color; accompanied by pain or pulling axillary pain. Increase in size or pain during menstruation and emotional changes are also the main symptoms of HMG. In severe cases, it can cause acute mastitis and risk of breast cancer and affect normal work and life. With the progress of society, women's work and lifestyle has dramatically changed, resulting in hormonal disorders in the body, so that the prevalence of breast hyperplasia is increasing year by year [2].

There are several treatment options available for managing breast hyperplasia. Hormone therapy and endocrine therapy are mainly used in the treatment of this disease by Western medicine, but the efficacy is not satisfactory and long-term hormone therapy may increase the developing of cancer [1]. Mongolian medicine RuXian-1 is a traditional drug formula for the treatment of mammary hyperplasia which has been widely used in clinic in Inner Mongolia Autonomous Region [3,4]. However, the mechanism of RuXian-1 improves breast hyperplasia is not yet clear. Current research has found that the mechanism of RuXian-1 in the treatment of breast hyperplasia may be related to the regulation of sex hormone levels, sex hormone receptors and their mRNA expression, and these factors may participate in the regulation of cell proliferation and apoptosis, and DNA synthesis and serum lipids [5]. Furthermore, Wang et al. [6] and others found that RuXian-1 has potent effect to breast hyperplasia by inhibiting the expression of the anti-apoptotic protein Bcl-2 and increasing the expression of the pro-apoptotic protein Bax. However, the study of Liu Lantao found that RuXian-1 inhibited the expression of Bax protein. This suggests that the effect of RuXian-1 on breast hyperplasia may regulated by other mechanisms [7].

Glucose-regulating protein 78 (GRP78) is a molecular chaperone in the endoplasmic reticulum (ER) and plays an important role in maintaining protein stability, regulating protein folding and assembly of proteins. The GRP78 has been reported upregulated in many different types of cancers, including breast cancer. It is known that GRP78 is important protein for mammary gland development, however, higher levels of GRP78 protein may correlated with ER stress-induced breast hyperplasia [8,9].

PERK is a serine threonine kinase, which is activated by autophosphorylation and dimerization after disaggregation from GRP78. Activation of PERK leads to attenuation of mRNA translation, protecting cells from the influx of misfolded proteins to cut down protein load in the ER and rebuild the homeostasis of endoplasmic reticulum (ER) [10]. Early in the endoplasmic reticulum stress (ERS), the activated PERK signaling pathway can protect cells by inhibiting protein synthesis. However, prolonged ERS triggers apoptosis, not directly, but by activating downstream proapoptotic molecules that push cells toward the death pathway. The expression of CHOP is of great significance in inducing prosurvival to proapoptosis [10].

C/EBP homologous protein (CHOP), also known as GADD153, belongs to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors that have been implicated in the regulation of processes relevant to cellular proliferation, differentiation, and expression, and energy metabolism of cell type-specific genes [11,12]. CHOP is a 29 kDa protein with 169 (human) or 168 (rodents) amino acid residues. CHOP is generally induced by genotoxic stress and growth arrest signals [13]. increasing evidence has suggested that numerous diseases are relevant to the abnormal expression of CHOP in ER stress, Included in the HMG [14]. Thus, in the present study, we investigated the effect of the traditional Mongolian drug RuXian-1 on protein expression levels of GRP78 and its target gene, CHOP using in a rat model of breast hyperplasia.

1. Materials and methods

1.1. Materials and drug preparation

Estradiol benzoate injection was purchased from Hangzhou Animal Pharmaceutical Co., Ltd. (Hangzhou, China). Progesterone injection was purchased from Zhejiang, Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). Tamoxifen was obtained from Shandong Jiankang Co., Ltd. (Shandong, China). Polyclonal primary antibodies for GRP78, CHOP, PERK, and secondary antibody for horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were obtained from Shanghai Univision Bio-technology Co. (Shanghai, China). Mongolian RuXian-1 (Lot No: Zhe Wei 9604-79) was provided by Affiliated Hospital of Inner Mongolia Minzu University's. Mongolian Medicine Dispensing Room. The administration dosage to rats were converted from the clinical drug dose of patients.

1.2. Development of rat breast hyperplasia model and drug treatment

Laboratory Animals and feeding environment: Female non-pregnant Wistar rats were purchased from Changchun Yisi Laboratory Animal Co. (Changchun, China; approval No. SCXK-Ji-2018-0007). The body weight is all in the 200–220 g range. The mice were maintained in Inner Mongolia University for Nationalities Experimental Animal Facility under specific pathogen-free conditions with ad libitum access to standard water and food and with a 12-hour light-dark cycle (room temperatures 18–22 ℃ with 40%–70% humidity). The animal experiments were approved by the Ethics Review Committee of Inner Mongolia Minzu University (NM-LL-2018-12-18-04).

Animal group: Wistar rats were weighed and grouped according to the randomized numerical method after being kept in the above environment for one week. According to each group of 8 rats, they were divided into Normal group (negative control: non-treatment group), Model group (Estradiol benzoate-treatment group), Positive drug group (Tamoxifen group), and RuXian-1-treatment group. The modeling of mammary hyperplasia was performed and adopted from the 2017 Chinese Society of Traditional Chinese Medicine's Specification for the Preparation of Animal Models of Mammary Hyperplasia Disease; Wistar rats were injected with estradiol benzoate at 0.5 mg/kg once daily intramuscularly for 25 days, followed by progesterone at 4 mg/kg once daily intramuscularly for 5 days. Five modeling rats were randomly selected on day 31 to test hormone levels and pathological changes to verify modeling.

Drug dosage: After successful developed the hyperplasia model, the rats were administered with RuXian-1 and tamoxifen once daily by oral gavage at 3.0 g/kg and 1.8 mg/kg for 4 weeks, respectively. The normal group rats given water once daily by oral gavage. On day 29, blood sample was removed, and mammary gland tissue was taken and fixed with 4% paraformaldehyde.

1.3. Immunohistochemical assay

The fixed mammary gland tissues were dehydrated and embedded in paraffin and cut into sections of 5–10 μm. The sections were incubated in peroxidase blocking reagent (3% H_2O_2 solution) for 10 min. Next, the sections were incubated with primary

antibodies (GRP78, CHOP, and PERK) for 2 h at room temperature. After washed 3 times with phosphate-buffered saline (PBS), the sections were incubated with secondary antibody with HRP-labeled IgG for 30 min at room temperature. Washed again with the PBS the sections were then stained with 3, 3′-Diaminobenzidine (DAB) staining reagent. The images of DAB-stained tissue were visualized by microscopy.

1.4. Western blotting assay

Rat mammary tissue was cut into small pieces and homogenized with RIPA lysis buffer, and then centrifuged at 4 ℃ for 10 min at 12,000 rpm to remove the supernatant. Protein concentrations of samples were determined by using *bicinchoninic acid* (BCA) protein assay kit. The protein samples were mixed with sample buffer and loaded onto 8% SDS-PAGE gel, and transferred to polyvinylidene fluoride (PVDF) membranes (Immun-BlotTM, Bio-Rad). The membranes were then blocked with 4% (w/v) nonfat dry milk powder in 1 × tris-buffered saline with tween (TBST) buffer (Santacruz Biotechnology, Texas, USA). The membranes were incubated with primary antibodies of GRP78, PERK, and CHOP at 4℃ overnight and washed three times with washing buffer. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated corresponding secondary antibodies for 1 h, followed by washing again with washing buffer. The proteins were visualized using the enhanced chemiluminescence (ECL) detection reagent (Waka, Osaka, Japan). Images of protein taken with the ImageQuant machine. The intensity of each band was quantified using ImageJ software V1.8.0 (NIH, Bethesda, USA). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for endogenous control. Three independent experiments were conducted.

1.5. Statistical analysis

Data were analyzed with the IBM SPSS Statistics® version 17 (IBM Corp, Armonk, NY, USA). All values are expressed as the means ± SD. One-way analysis of variance (ANOVA) test was used to determine whether the differences among multiple groups were significant. Differences were considered statistically significant at $P < 0.05$.

2. Results

2.1. Effects of Mongolian drug RuXian-1 on the expression level of GRP78 protein in rat mammary gland tissue

As shown in **Figure 1**, the immunohistochemical and western blotting results showed that the expression level of GRP78 protein in mammary gland tissue from estradiol benzoate-treatment group was lower than that in normal group. In comparison to estradiol benzoate-treatment group, RuXian-1 treatment significantly upregulated the protein expression level of GRP78. The induction of GRP protein is higher in RuXian-1 group than Tamoxifen group (positive control), suggesting that RuXian-1 more effectively upregulate the expression level of GRP78 in mammary gland tissue.

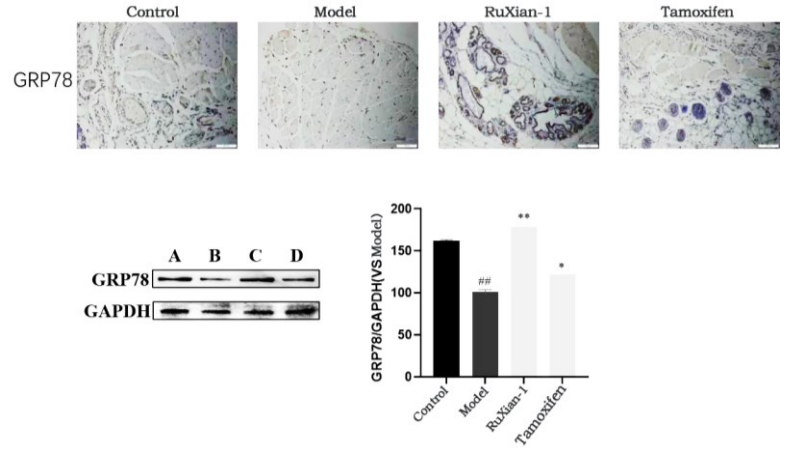

Figure 1. Representative images of GRP78 protein by IHC (A) and western blotting (B) analyses, quantitative analyses of western blotting results (C).
**P* < 0.05, ***P* < 0.01, ##*P* < 0.01.

2.2. Effects of Mongolian drug RuXian-1 on the PERK signaling pathway

As shown in **Figure 2**, the immunohistochemical and western blotting results showed that the expression level of PERK protein in mammary gland tissue from estradiol benzoate-treatment group was lower than that in normal group. In comparison to estradiol benzoate-treatment group, RuXian-1 significantly upregulated the protein expression level of GRP78, but not Tamoxifen.

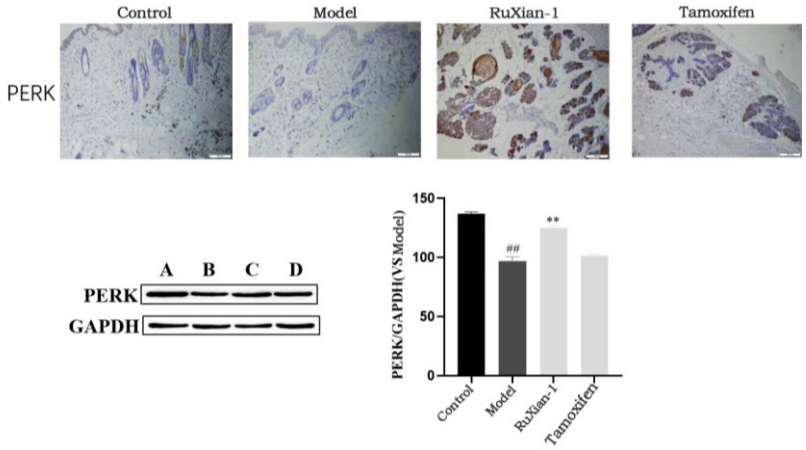

Figure 2. Representative images of PKR-like endoplasmic reticulum kinase (PERK) protein by IHC (A) and western blotting (B) analyses, quantitative analyses of western blotting results (C).
***P* < 0.01, ##*P* < 0.01.

2.3. Effects of Mongolian drug RuXian-1 on the GRP78 target gene, CHOP

As shown in **Figure 3**, the immunohistochemical and Western blotting results showed that the expression level of CHOP protein in mammary gland tissue from estradiol benzoate-treatment group was lower than that in normal group. In comparison to estradiol benzoate-treatment group, RuXian-1 significantly upregulated the protein expression level of GRP78. It is known that both of GRP78 and CHOP are

commonly used as markers of endoplasmic reticulum (ER) stress, involved in cell proliferation, invasion. As an ER chaperone, GRP78 functions as a potent anti-apoptotic factor. In our results, E2 significantly inhibit the expression of GRP78 and CHOP in rat mammary gland tissue. Importantly, RuXian-1 significantly upregulated the GRP78/PERK/CHOP expression, which induces apoptosis and normally acting to inhibit over proliferation of cells.

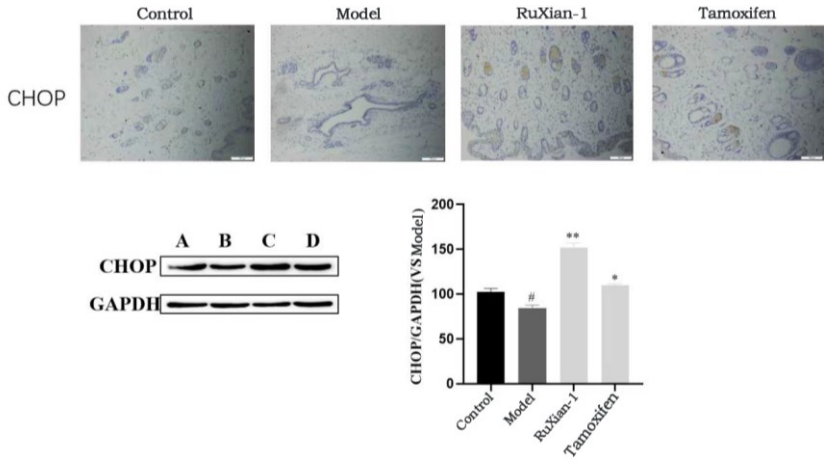

Figure 3. CHOP expression in breast tissue of each group. A, B, C and D are the normal group, the model group, the mast-i treatment group and the tamoxifen treatment group compared vs. normal group, respectively.
##$P < 0.01$; vs. model group. *$P < 0.05$, **$P < 0.01$.

3. Discussion

Breast hyperplasia is the most common benign breast disease in clinic, the clinical manifestation is breast pain, nodular breast, or palpable breast lumps, with nipple discharge in some patients. Hyperplasia of mammary gland is not tumor; however, it is an important risk factor for breast cancer [2]. The treatment of breast hyperplasia with Chinese medicine is becoming more and more widely used in clinical practice, and its mechanism of action is multi-targeted and multi-directional. RuXian-1 is a traditional Mongolian herbal medicine and specifically used for breast hyperplasia. The formula consists of 30 herbs, including cardamom, motherwort, deer antler, cordyceps sinensis and sea buckthorn. RuXian-1 is based on the theory of Yin-Yang and five elements, six flavors, and eight natures in Mongolian medicine. The effectiveness and safety of RuXian-1 in the treatment of breast hyperplasia has been confirmed many times in the clinic and in animal experiments, but its mechanism of action is not clear. Current research has found that the mechanism of RuXian-1 in the treatment of breast hyperplasia may be related to the regulation of sex hormone levels, sex hormone receptors and their mRNA expression, and modulates the cell proliferation and apoptosis [5]. However, the expression of the apoptotic protein BCL2-Associated X (Bax) is controversial, maybe Bax-independent signaling pathways that are involved in the therapeutic effect of RuXian-1. We therefore investigate the possible molecular mechanism of RuXian-1 on mammary gland tissue.

Endoplasmic reticulum stress (ER stress) is a pathological state in which the physiological function of the endoplasmic reticulum is disturbed, a self-defense

mechanism of the organism, and excessive ER stress will lead to apoptosis [15]. In recent years, the endoplasmic reticulum stress pathway has attracted much attention as a regulator of cell proliferation and apoptosis in multiple tissues, including breast tissue [16]. Unfolded protein response (UPR) is a stress response that is specific to the ER stress. The GRP protein protects cells from stress induced by the accumulation of unfolded proteins in the endoplasmic reticulum under stress condition. Furthermore, ER stress mediated by GRP78/PERK/CHOP signaling is directly involved in cellular apoptosis. It is known that GRP78 activates PERK signaling to upregulate CHOP and cellular apoptosis.

In this study, the expression of GRP78, PERK and CHOP was down-regulated in rat treatment with estrogen and progesterone, but increased after treatment with RuXian-1, suggesting that RuXian-1 promotes the activation of GRP78/PERK/CHOP, which promotes the apoptosis of hyperplastic mammary cells.

Author contributions: Conceptualization, SZ; methodology, JJ; software, AT; resources, CC; data curation, AT; writing—original draft preparation, JJ; writing—review and editing, SZ. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

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