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Original Article

Experimental study on the clinical effects of Xiaoru Sanjie Jiaonang on mammary glands hyperplasia and ki-67

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Abstract: Objective: This study aims to observe the effect and mechanism of Xiaoru Sanjie Jiaonang (XRSJ) on the treatment of mammary gland hyperplasia, and provide a theoretical basis and clinical evidence for clinical expansion. Methods: Japanese white rabbits were randomly divided into three groups: high-, middle- and low-dose groups; Xiaoyao Pill group; model control group; normal control group. The observation points were as follows: before XRSJ administration, three months after XRSJ administration, and three months after XRSJ discontinuance. Changes in breast height, morphological changes of the mammary gland under a light and electron microscope, and the expression of ki-67 were observed. At the same time, patients diagnosed with mammary gland hyperplasia at an Outpatient Clinic were selected and divided into treatment groups. These patients received XRSJ and Xiaoyao Pills, respectively, for one month, while patients in the control group did not receive any drug treatment. Clinical efficacy was observed while rechecking at the Outpatient Clinic after three months. Treatment with a therapeutic dose of XRSJ could significantly reduce breast height, decrease the number of lobules and acini in hyperplastic mammary glands and the layer number of ductal glandular epithelial cells, substantially lower the content of serum estradiol (E2), significantly downregulate the expression of ki-67 protein in mammary tissues, and inhibit mammary gland hyperplasia. Conclusion: XRSJ treatment can relieve mammary tissue hyperplastic lesions, reduce E2 levels and downregulate the expression of ki-67. It has a significant therapeutic effect on mammary gland hyperplasia.

Keywords: Xiaoru Sanjie Jiaonang (XRSJ), mammary gland hyperplasia, ligustilide

Introduction

At present, people are increasing being affected by breast disorders. Among these, hyperplasia of the mammary gland is one of the most common benign diseases of the breast in middle-aged women [1], in which hundreds of millions of Chinese patients suffer from breast hyperplasia. From the clinical point of view of traditional Chinese medicine, this is classified under “Rupi” [2], which accounts for more than 70% of all breast diseases [3]. A report revealed that hyperplasia of the mammary gland, particularly atypical hyperplasia, is a potential precancerous lesion. Some of these can be transformed into breast cancer, which seriously threatens the general physical and mental health of women [4, 5]. Therefore, the treatment of this disease has important clinical value [6, 7].

On one hand, the aberrant proliferation and migration of tumor cells have become a hallmark of tumor pathology during tumor progression. The abnormal expression of cell factors plays an important role in tumor cell proliferation and migration, and provides a clinical basis for the diagnosis, treatment and prognosis of tumors [8]. Ki-67 is a gene located in chromosome 10, which can be detected in the proliferating cell nuclear matrix. Ki-67 expression and its function in luminal breast cancer have not been completely elucidated. The results of the present study revealed that Ki-67 is associated with mitosis, and is especially closely associated with tumor cell proliferation [9-11].

On the other hand, Omar Hameed studied a number of patients with hyperplasia of the mammary gland, and found that the expression

of ki-67 was associated with atypical hyperplasia. Furthermore, some researchers have conducted further trials, their findings suggest that the high expression of Ki-67 plays a significant role in the promotion of the pathogenesis and development of breast cancer. In addition, Ki-67 plays crucial roles in promoting the genesis and metastasis of breast cancer [12].

Some patients with breast dysplasia were treated by surgical treatment. However, the surgery would leave a scar, and the recurrence rate is extremely high. This would seriously affect the physical and mental health of women [13]. From the perspective of humanistic medicine, the psychological burden of scarring after mammary hyperplasia surgery is far greater than the disease itself, especially for young patients. Therefore, searching for effective and non-invasive treatment methods for breast hyperplasia is particularly important.

The aim of the present study was to investigate the effect of XRSJ on mammary gland hyperplasia and the expression of Ki-67, as well as its possible inhibitory effect on the precancerous lesions of breast cancer.

Materials and methods

Experimental animals

Female Japanese white rabbits were used for the present study. These rabbits were not pregnant, weighted 2.1 ± 0.1 kg, were five months old, and were housed individually, given drinking water and fed with complete pellet feed (the provided white rabbits were fed by the Experimental Animal Center of The Second Affiliated Hospital of Harbin Medical University). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Medicines

Xiaoru Sanjie Jiaonang (XRSJ): Shandong Shenzhou Pharmaceutical Co., Ltd. (SFDA approval no. Z20027142). Main ingredients: bupleurum (wax for broiling), angelica, edible tulip, etc. Diethylstilbestrol injection: Shanghai General Pharmaceutical Co., Ltd. (SFDA approval no. H31021403). Progesterone injection: Shanghai General Pharmaceutical Co., Ltd. (SFDA approval no. H31021401). Xiaoyao Pill: The Third Harbin Chinese Medicine Plant of Harbin Pharmaceutical Group (SFDA approval no. Z23022068). Lidocaine: Shanghai Fosun Zhaohui Pharmaceutical Co., Ltd. (SFDA approval no. H31021071).

Experimental methods and procedures

Establishment of the mammary gland hyperplasia model: A total of 90 rabbits were used in the present study. These rabbits were randomly divided into six groups (n=15, each group): A group, high-dose of XRSJ; B group, middle-dose of XRSJ; C group, low-dose of XRSJ; D group, Xiaoyao Pill; E group, model group; F group, normal controls. Rabbits in the first five groups were intramuscularly injected with 0.2 mg/kg×d of diethylstilbestrol for 25 consecutive days, and injected with 1 mg/kg×d of progesterone for five consecutive days to establish the mammary gland hyperplasia model. Rabbits in the F group were intramuscularly injected with the same dose of saline for 30 consecutive days [14].

Method of administration: After the modeling was completed, rabbits in the A, B and C groups were intragastrically administered with 0.30, 0.10 and 0.04 g/kg of XRSJ, twice a day, rabbits in the D group were administered with 0.20 g/kg of Xiaoyao Pill, twice a day, and rabbits in the E and F groups were administered with an equal dose of distilled water, twice a day. Rabbits in each of the groups above were intragastrically administered with the treatment for three months. The dose used in the present study was calculated based on the conversion relation between humans and rabbits [14].

Sampling and test: The first to the third pair of breasts were orderly cut under local anesthesia (lidocaine) for three times before the administration of XRSJ, three months after the administration of XRSJ, and three months after the discontinuance of XRSJ. Each pair of breasts were respectively fixed with 10% formaldehyde, embedded in paraffin, sliced, and stained with hematoxylin and eosin (H&E) for observation under light microscopy. Then, the specimens were fixed with 2.5% glutaraldehyde, embedded with Epon 812, sliced in ultra-thin sections, and stained with acetic acid dioxygen oil and lead citrate for observation under an electron microscope. Next, the content of Ki-67 protein in mammary gland cells was determined by western blot. A vernier caliper was used to mea-

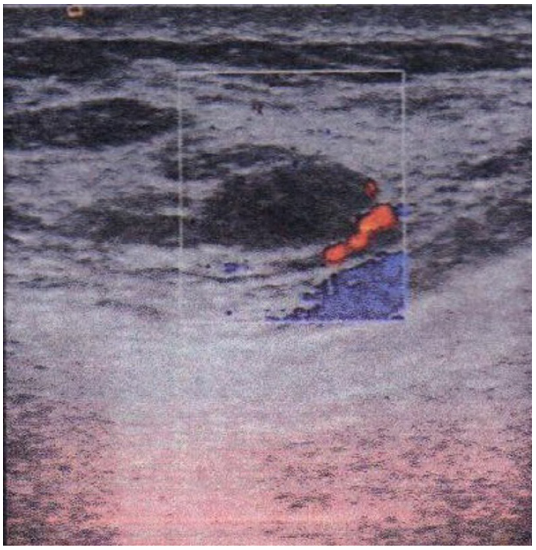

Figure 1. The appearance of hyperplasia of mammary gland in ultrasonography. In the figure, the black space in the box is a hyperplasia of the mammary gland.

sure the height of the second pair of breasts before and after the administration of the treatment. Heart blood samples were collected while cutting the breast. Radioimmunoassay was used to examine the serum E2 level.

Clinical application

Case selection: Patients diagnosed with mammary gland hyperplasia at the Outpatient Clinic was the object of study [1]. These patients had positive signs of stabbing pain and proliferative nodules of more than 0.5 cm (**Figure 1**).

Inclusion criteria

Inclusion criteria: female patients who were 20-40 years old; Patients who did not receive other drug treatments before seeing the doctor; patients who completed the course of treatment. (A total of 745 female patients who met the above diagnostic criteria were selected for the study. Among these patients, 611 patients met the inclusion criteria, and the remaining 134 patients did not follow the medication regimen or suspended the medication).

Treatment protocol

The above patients were divided into three groups: group A, patients who received XRSJ (241 patients); group B, patients who received the Xiaoyao Pill (192 patients); group C: untreated patients (178 patinets). The treatments for the above patients were all orally administered according to instructions, and one month was equivalent to one course of treatment. Breast tenderness, hypertrophic nodules >0.5 cm, and the relationship with menstruation were recorded before and after the treatment. These patients revisited the hospital and were examined by B-mode ultrasound three months later.

Efficacy evaluation

Effective: Physical examination: Breast symptoms of stabbing pain weakened or disappeared, and the hyperplasia range significantly narrowed according to B-mode ultrasound results (hyperplastic nodules >0.5 cm shrank or disappeared).

Ineffective: Physical examination: Breast symptoms of stabbing pain had no relief, and the hyperplasia aggravated according to B-mode ultrasound (the hyperplasia had no relief, or became bigger and harder).

Statistical methods

SPSS 12.0 software was used to statistically analyze the experimental data, and all the data were expressed as $\bar{x} \pm$ standard deviation (SD).

Results

Changes in breast height

After three months of intragastric administration, breast heights in the A and B groups were significantly reduced ($P<0.05$). Compared with the F group, the difference was not statistically significant ($P>0.05$). Furthermore, there was no significant difference in breast height between the C and E groups, and between the D and E groups ($P>0.05$, **Figure 2**). The curative dose of XRSJ had a marked inhibitory effect on mammary gland hyperplasia.

Morphological changes in mammary tissues

Light microscopy examination: It was observed that mammary tissues in the F group had no hyperplastic lesion at 3× magnification by light microscopy. In the E group, typical mammary gland hyperplasia was found at 3× magnification by microscopy, which manifested as a significant increase in the number of lobules aci-

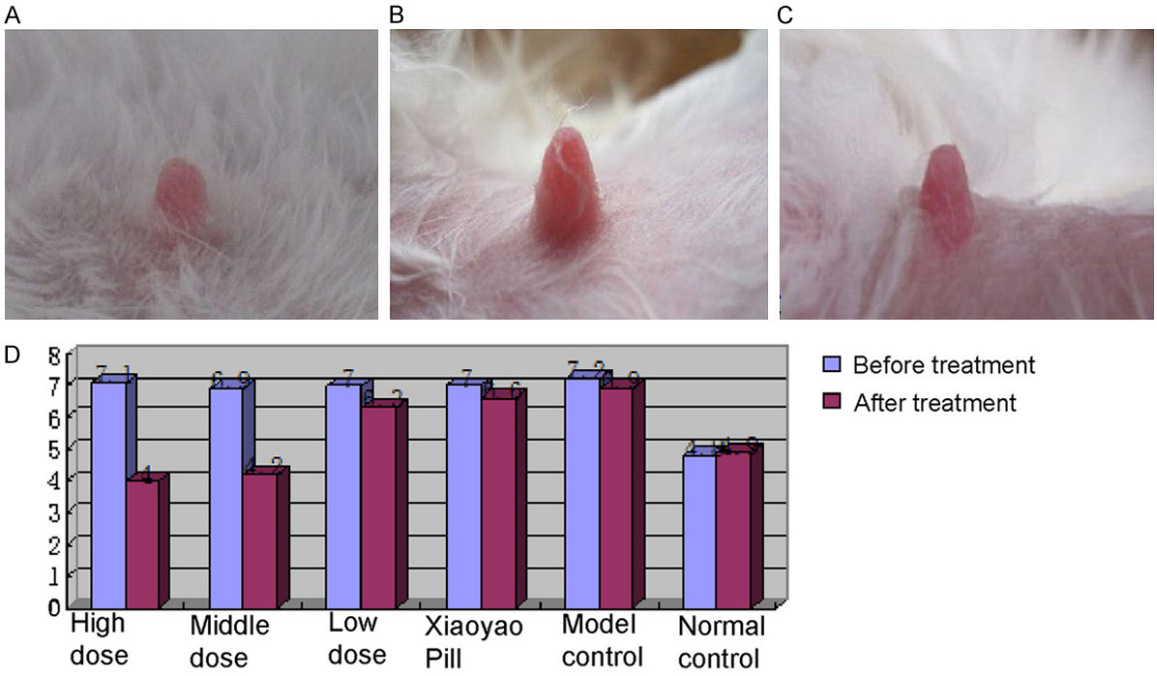

Figure 2. A: Breasts of normal control group F (2×); B: Breasts of model group E (2×); C: Breasts of Group A after three months of treatment (2×); D: After 3 months of intragastric administration, XRSJ high and medium dose groups showed a significant reduction in breast height, 3.1 mm in the high dose group, and 2.7 mm in the medium dose group P<0.05; The other groups did not significant change *P>0.05.

nus in the hyperplastic mammary glands, the layer number of ductal glandular epithelial cells, and the number of connective tissues and blood capillaries (**Figure 3**). In the A and B groups, these three indicators of mammary gland hyperplasia (the number of lobules acinus in the hyperplastic mammary glands, the layer number of ductal glandular epithelial cells and the number of connective tissues and blood capillaries) significantly decreased after treatment, compared with that before treatment and with the E group, and the difference was statistically significant ($P<0.05$). However, there was no significant difference when compared with the F group ($P>0.05$). These breast tissues were almost normal. In the C and D groups, there was no significant treatment effect, and the hyperplasia of the mammary gland was obvious at 3× magnification by microscopy.

*Examination by electron microscopy (**Figure 4A-C**):* The glandular epitheliums in the F group presented as monolayer cubes, were normally arranged, and had a uniform basement membrane. Gland cells in the E group were multilayered and scale-arranged. In addition, the cell polarity disappeared and the papillae appeared. Furthermore, the number of mitochondria in the cytoplasm increased, in which the Golgi and rough endoplasmic reticulum were abundant. Moreover, abundant glycogen granules were found in the cell nucleus. After treatment with XRSJ, the glandular epitheliums in the A and B groups were monolayered and arranged into a high columnar shape, the nucleus moved upwards, and the ductal glandular cavity tightened. Compared with that before treatment and with the E group, the number of mitochondria, Golgi and rough endoplasmic reticulum significantly decreased ($P<0.05$, **Figure 4D**). However, part of these hyperplastic cells died. In the A and B groups, the treatment effect was maintained for three months after the treatment was discontinued, and there was no significant difference when compared with three months after the administration of treatment ($P>0.05$). The difference in treatment effects in the C and D groups was not statistically significant.

Serum sexual hormone levels

The levels of serum E2 in the A-E groups were all significantly higher than those in the F group ($P<0.01$). After treatment, serum E2 values in the A, B and D groups decreased, and there

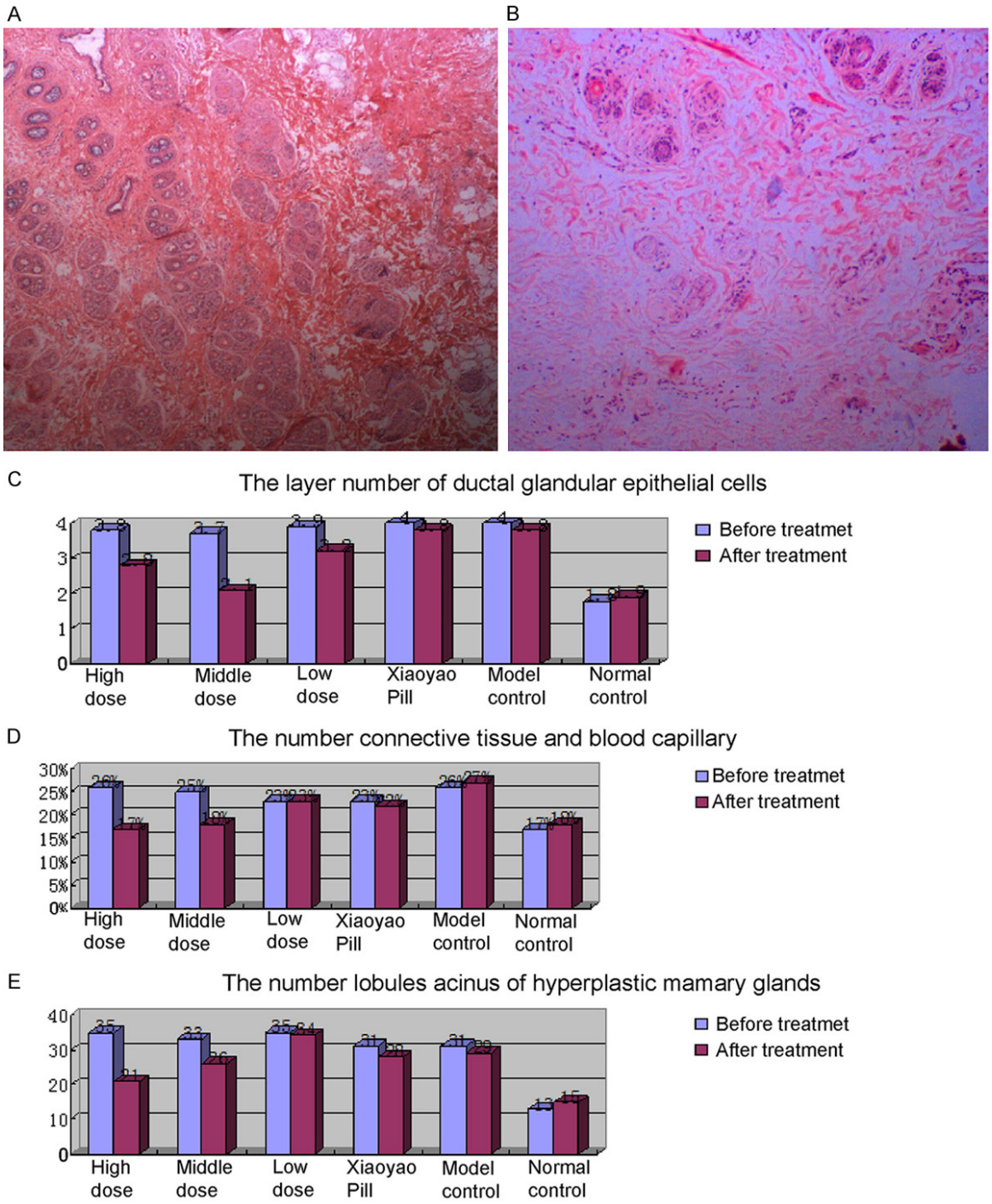

Figure 3. A: The acini of model group E in light microscopy (100×); B: The acini of the high dose XRSJ at the end of treatment light microscopy (100×); C-E: The data in this figure are average values measured by 100× light microscopy. After treatment with XRSJ, the three indicators of mammary gland hyperplasia, such as the number of acini, the number of ductal glandular epithelial cells, the number of connective tissue and capillaries, were significantly decreased compared with those before treatment (P<0.05); Other groups did not change significantly *P>0.05.

was no significant difference in serum E2 values at three months after the administration of treatment, when compared with the F group (*P*>0.05). Furthermore, E2 values were all significantly lower than that in the E group (*P*< 0.01). Serum E2 values in the A, B and D groups were stable, and there was no recurrence at three months after discontinuing the drugs.

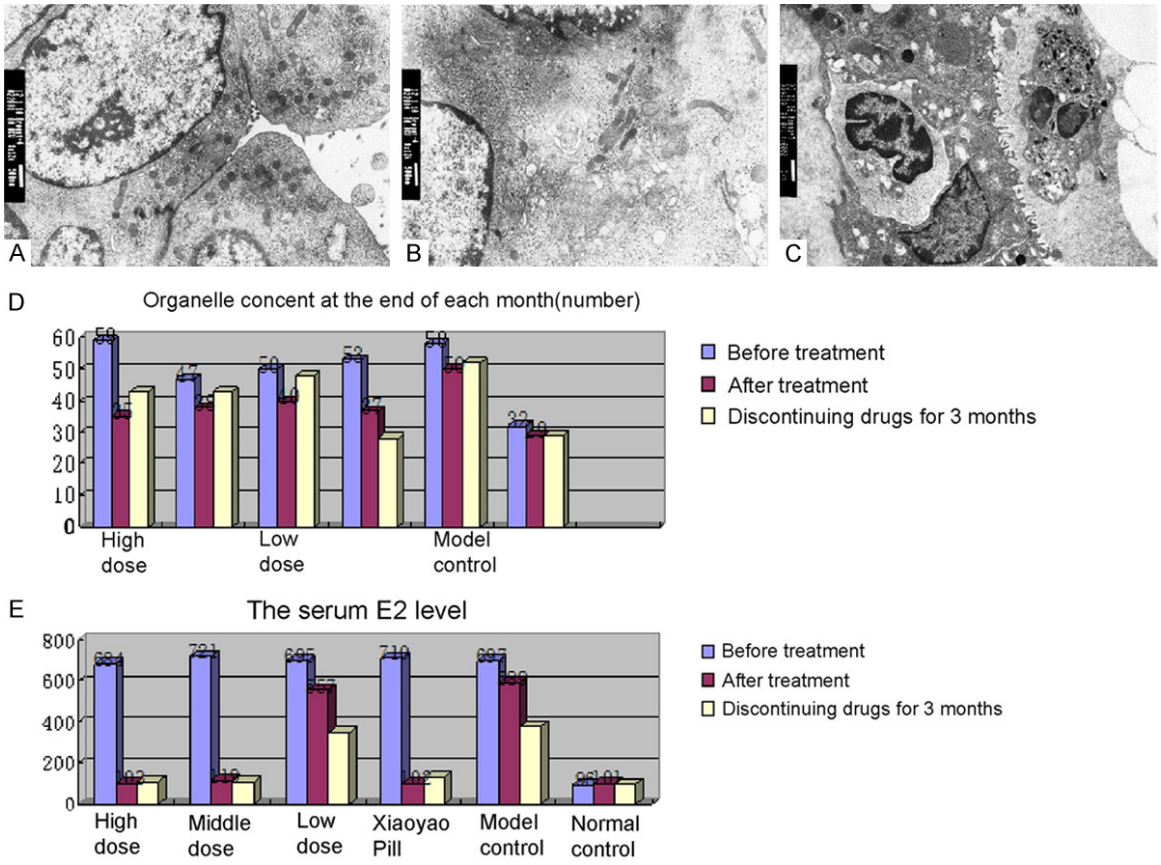

Figure 4. A: Glandular epithelial cells of model group E: glandular cells were stratified, squamous arrangement, the cell polarity disappeared, showing papillary processes; B: Glandular epithelial cells of Group A three months after the treatment; C: Apoptotic cells of Group A three months after the treatment; D: Organelle content after the treatment discontinue drugs for 3 months. The data in this table are average values measured by 12000× electron microscopy. The organelle content refers to the total number of mitochondria, Golgi apparatus and rough endoplasmic reticulum. A, B and D group, the number of organelles decreased significantly *P<0.05, and three months after stopping the breast cell proliferation still has a role. C group has a certain effect, but relapse after stopping. There was no significant change in E and F groups. E: The serum E2 level in before, at the end of and 3 months after the treatment. A, B and D groups after treatment, the serum E2 decreased, and serum E2 level showed after stable 3 months. Serum E2 value has decreased in C group. There was no significant change in E and F groups.

The difference was not statistically significant when compared with that at the end of treatment (P>0.05). Moreover, in the C group, the difference in serum E2 values before and after treatment was not statistically significant (**Figure 4E**).

*Content changes in Ki-67 protein in mammary gland hyperplasia cells (**Figure 5A** and **5B**)*

The expression of Ki-67 in mammary tissues in the F group were significantly lower than that in the E group (P<0.05). The expression of Ki-67 in the A and B groups decreased after treatment. There was a significant difference when compared with the E group (P<0.05), while there was no significant difference when compared with the F group (P>0.05). Furthermore, there was no significant difference in Ki-67 expression between A and B groups, and between the D and E groups (P>0.05).

Clinical effects

Three groups of patients received one month of treatment, and rechecked at the Outpatient Clinic at three months after completing the treatment. These patients had breast symptoms of stabbing pain, which existed after receiving the corresponding treatment: treated with XRSJ, 52 patients; treated with Xiaoyao Pills, 93 patients; untreated patients, 135 patients (**Figure 5C** and **5D**). Furthermore, the following patients presented with hyperplastic nodules >0.5 cm

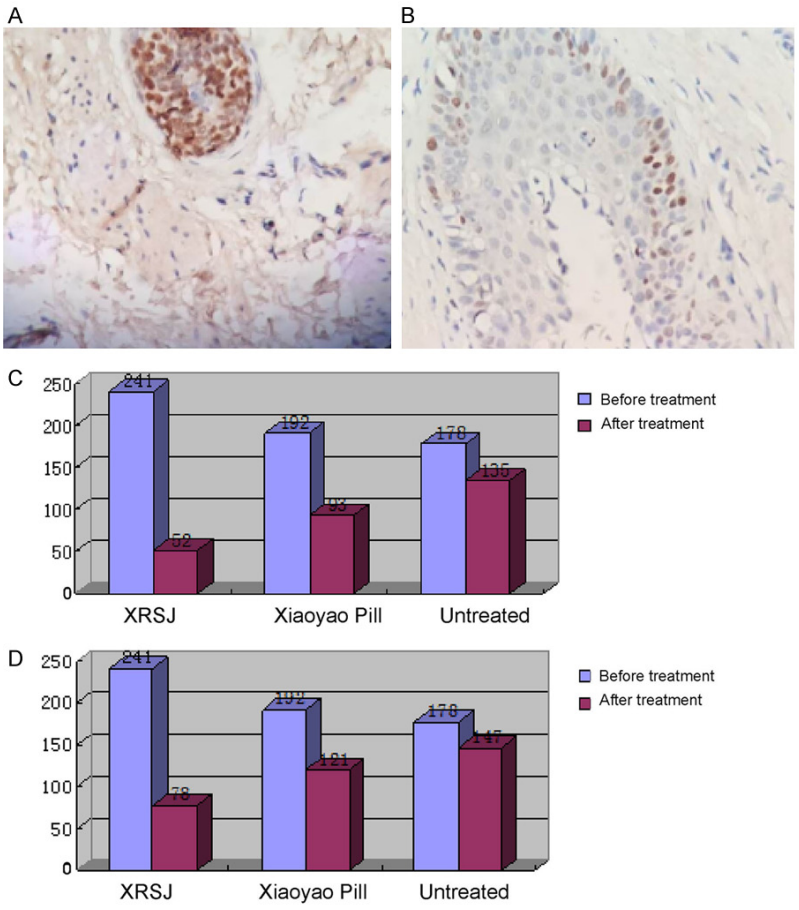

Figure 5. (A, B) The expression of Ki-67 protein was measured by immunohistochemical method, Rabbit breast tissue was stained with goat antibody, and the expression of Ki-67 in group A was compared before and after treatment. (A) is a pre-treatment image; (B) is a 3-month post-treatment image. Positive IHC signals were visualized with brown color and do not stain or stain for the negative. (C, D) Outpatient group of patients with breast hyperplasia a total of 611 people dividing them into three groups receiving XRSJ, Xiaoyao Pill and no treatment, the treatment time was one month, three months after the completion of treatment in outpatient review. After treatment, the symptoms of breast sting still exist: taking Xiaogan Sanjie: 52 cases; Xiaoyao Wan: 93 cases; not used: 135 cases (C); Patients after treatment >0.5 cm proliferative nodules exist: taking Xiaogan Sanjie: 78 cases; Xiaoyao Wan: 121 cases; untreated: 147 cases (D).

after receiving the corresponding treatment: treated with XRSJ, 78 patients; treated with Xiaoyao Pills, 121 patients; untreated patients, 147 patients.

Experimental summary

Compared with Xiaoyao Pills, XRSJ could significantly inhibit mammary gland hyperplasia in rabbits, reduce breast height, decrease the number of lobules acinus in hyperplastic mammary glands and the layer number of ductal glandular epithelial cells, and reduce serum E2 and Ki-67 content. Both Xiaoyao Pill and XRSJ could reduce serum E2, and there was no significant difference in the reduced E2 levels between these two treatments. However, the therapeutic dose of XRSJ had a longer and more stable effect in reducing serum E2 levels without recurrence, when compared with that of Xiaoyao Pills. These animal experiments reveal that XRSJ could significantly inhibit hyperplasia in mammary gland cells, inhibit mammary gland hyperplasia, reduce Ki-67 and prevent precancerous lesions.

Discussion

Mammary gland hyperplasia is a kind of endocrine disorder mainly caused by an imbalance in the hypothalamus-pituitary-ovarian endocrine axis. The increase in E2 secretion, the relative reduction in P secretion, and the quality and quantity of abnormalities of E2 and P receptors all result in excessive mammary gland hyperplasia and subinvolution. These factors are the key to the onset of the disease [15]. Mammary gland hyperplasia has been recognized as one of the precancerous lesions of breast cancer. Therefore, the treatment of mammary gland hyperplasia can not only alleviate its symptoms and signs, but also play a role in preventing breast precancer. Therefore, XRSJ has important clinical significance on the treatment of this disease.

Chinese medicine attributes mammary gland hyperplasia into the category of “nodules of the breast”. In modern medicine, only few drugs can be used for the treatment of mammary gland hyperplasia. XRSJ has been used clinically for many years, which has shown marked effects. The investigator of the present study

has previously performed similar studies, which demonstrated that XRSJ can alleviate mammary gland hyperplasia and decrease the level of E2 [13]. In a study conducted by Li Jingwei *et al.* on the effect of Yanghehuayan Decoction on breast precancer and Ki-67 expression in rats, it was revealed that Yanghehuayan Decoction has a certain prevention effect on breast cancer in rats [14]. This product also obtained a conclusion similar to the Yanghehuayan Decoction, exhibiting that the product also had the ability to reduce the content of Ki-67 in mammary gland cells, and may have a certain degree of inhibition on precancerous lesions. Some scholars have observed the therapeutic effect of tea polyphenols on mammary gland hyperplasia in model rats, and considered that tea polyphenols had a certain therapeutic effect on mammary gland hyperplasia in rats. However, further studies are needed to determine whether this could have any effect on Ki-67 levels in mammary gland cells [15].

At present, Western medicine treatments for mammary gland hyperplasia and breast precancer mainly rely on tamoxifen. However, this drug has adverse reactions. Tamoxifen can inhibit the effect of estrogen on the bone, endometrium and vaginal epithelium, and have some effect on liver function [16]. In addition, adverse reactions are unavoidable when using tamoxifen for patients with mammary gland hyperplasia.

In the present study, XRSJ was intragastrically administered to treat a mammary gland hyperplasia model for rabbits for three months, and a six-month monitoring program was performed. Three observation points were set to observe the difference between groups and within groups: before the administration of XRSJ, three months after the administration of XRSJ, and three months after the discontinuance of XRSJ. The pathological sections confirm that high- and middle-doses of XRSJ could reverse the morphological changes of mammary tissues induced by mammary gland hyperplasia, and induce these to return to normal (**Figure 2D**). The serum sexual hormone test confirmed that high- and middle-doses of XRSJ could significantly reduce E2 content in the body, increase P content, and eliminate the initial factors of mammary gland hyperplasia. At three months after the end of the intragastric administration, the observation indicators in the A and B groups had no significant changes, and the curative effects of XRSJ were stable without recurrence. In order to further explore the clinical efficacy of XRSJ in the treatment of mammary gland hyperplasia, the investigator treated patients with mammary gland hyperplasia (241 patients) with oral XRSJ for one month, and performed a follow-up at three months after the treatment. The results revealed that XRSJ had a therapeutic effect on mammary gland hyperplasia, could relieve the symptoms of breast pain and reduce or eliminate mammary mass, and had no obvious adverse reactions. Some scholars have conducted related studies and obtained a similar conclusion with this experiment [17, 18], proving that XRSJ had an exact curative effect on mammary gland hyperplasia.

XRSJ could significantly downregulate the activity of mammary gland hyperplasia cells. The sharp reduction in various organelles indicates that XRSJ slowed down the metabolism of glandular cells and inhibited their multipolar division. Partial hyperplastic cell apoptosis suggests that XRSJ could limit their growth and eventually eliminate hyperplastic cells (**Figure 4A-C**). Furthermore, XRSJ could significantly reduce the content of ki-67 in hyperplastic cells (**Figure 5A** and **5B**). Ki-67 can be used as an indicator to predict the incidence of breast cancer, and to evaluate the prognosis by measuring Ki-67 levels.

Ki-67 is a proliferative cell-associated nuclear antigen, and its function is closely correlated to mitosis. The abnormal expression of ki-67 leads to the rapid proliferation of mammary gland epithelial cells, and even the occurrence of breast cancer. Therefore, the downregulation effect of XRSJ on Ki-67 has significant clinical therapeutic value, which may have a certain effect on the inhibition of breast precancer [19-22]. The author considers that it may be closely associated with the ingredient of angelica in XRSJ. Angelica contains many lipid compounds such as ligustilide [23-25]. From the molecular point of view, ligustilide can significantly downregulate the intracellular expression of ROS, ERK and P38, thereby blocking the MAPK pathway, which is an important way for cell proliferation and differentiation. This would induce cells to stop at a stable G0/G1 stage from the active G1/S stage to stop mitosis [26-29], eventually

resulting in hyperplastic cell apoptosis. Some studies have also pointed out that ligustilide could induce the regulation of OGD/R, affect intracellular iron metabolism, and cause the formation of free-radicals in cells, oxidative stress and cell apoptosis [30]. However, the specific mechanism therein remains to be confirmed. At the same time, ligustilide has anti-inflammatory and neuroprotective effects (which improve activity and reduce inflammatory markers through its anti-oxidation and anticholinergic effects to some extent), and the main clinical manifestation of mammary gland hyperplasia-pain may also be alleviated through this approach [31, 32].

Conclusion

XRSJ can significantly reverse mammary gland hyperplasia and reduce E2 levels with stable efficacy, substantially downregulate the activity of mammary gland hyperplasia cells of rabbits, and partly cause hyperplastic cell apoptosis. It can also relieve breast pain, reduce the mass of hyperplasia, and may prevent breast precancer.

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Disclosure of conflict of interest

None.

Abbreviations

XRSJ, Xiaoru Sanjie Jiaonang; E2, serum estradiol.

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References

[1] Chen T, Li J, Chen J, Song H, Yang C. Anti-hyperplasia effects of Rosa rugosa, polyphenols in rats with hyperplasia of mammary gland. Environ Toxicol Pharmacol 2015; 39: 990-996.

[2] Zheng J, Zhao Y, Wang Y, Hu S, Lu P, Shen X. The infrared radiation temperature characteristic of acupoints of mammary gland hyperplasia patients. J Evid Based Complement Alternat Med 2013; 2013: 567987.

[3] Zhou LN. Chinese medicine treatment of breast hyperplasia. Zhong Yi Yao Dao Bao 2010; 16: 112-113.

[4] Wang L, Zhao D, Di L, Cheng D, Zhou X, Yang X, Liu Y. The anti-hyperplasia of mammary gland effect of Thladiantha dubia root ethanol extract in rats reduced by estrogen and progestogen. J Ethnopharmacol 2011; 134: 136-140.

[5] Zhang J, Rui X, Wang L, Guan Y, Sun X, Dong M. Polyphenolic extract from Rosa rugosa tea inhibits bacterial quorum sensing and biofilm formation. Food Control 2014; 42: 125-131.

[6] Liang Y, Deng D, Lai XJ, Tao J, Zhang XH, Chen Z, Zhao H. Clinical study on the diagnosis of hyperplasia of mammary gland by color doppler ultrasound. Xi Nan Guo Fang Yi Yao 2011; 21: 48-51.

[7] Chen X, Chen CC, Guo BL, Pang JW, Yang XW, Zhang DW, Yan CQ, Shen B, Zhong L, Qin YY, Zhang JG. The experimental study on the treatment of rabbit mammary hyperplasia with the treatment of xiaorusanjie capsule. Xian Dai Sheng Wu Xue Jin Zhan 2012; 12: 6249-6281.

[8] Sun Z, Shi Y, Shen Y, Cao L, Zhang W, Guan X. Analysis of different HER-2 mutations in breast cancer progression and drug resistance. J Cell Mol Med 2015; 19: 2691-2701.

[9] Beresford MJ, Wilson GD, Makris A. Measuring proliferation in breast cancer: practicalities and applications. Breast Cancer Res 2006; 8: 216.

[10] Miller HC, Drymousis P, Flora R, Goldin R, Spalding D, Frilling A. Role of Ki-67 proliferation index in the assessment of patients with neuroendocrine neoplasias regarding the stage of disease. World J Surg 2014; 38: 1353-1361.

[11] Rademakers SE, Hoogsteen IJ, Rijken PF, Terhaard CH, Doornaert PA, Langendijk JA, van den Ende P, van der Kogel AJ, Bussink J, Kaanders JH. Prognostic value of the proliferation marker Ki-67 in laryngeal carcinoma: results of the accelerated radiotherapy with carbogen breathing and nicotinamide phase III randomized trial. Head Neck 2015; 37: 171-176.

[12] Yuan P, Xu B, Wang C, Zhang C, Sun M, Yuan L. Ki-67 expression in luminal type breast cancer

and its association with the clinicopathology of the cancer. Oncol Lett 2016; 11: 2101-2105.
[13] Baker JL, Hasteh F, Blair SL. Atypical ductal hyperplasia at the margin of lumpectomy performed for early stagebreast cancer: is there enough evidence to formulate guidelines? Int J Surg Oncol 2012; 2012: 297832.
[14] Li JW, Liu XF, Chen HZ, Chen HH, Shi GX, Wang SJ. The effects of Yang and chemical rock decoction on the inhibition of precancerous lesions and the expression of ki67 in rats. Chinese Journal of Integrated Traditional and Western Medicine 2014; 34: 970-975.
[15] Yu LR, Ge WT, Li HX, Pan YH, Lv JH. The effect of tea polyphenol on the treatment of rats with hyperplasia of mammary gland. Chinese Journal of New Drugs and Clinical Remedies 2009; 28: 675-678.
[16] Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques. Breast Cancer Res Treat 1998; 48: 221-229.
[17] Wang YQ. A total of 102 cases of mammary gland hyperplasia were treated with the treatment of udder. Yi Xue Xin Xi 2010; 5: 539-540.
[18] He SJ. The clinical analysis of 200 cases of mammary gland hyperplasia was treated with the elimination of emulsion. Dang Dai Yi Xue Za Zhi 2009; 15: 153-154.
[19] Wang RX, Chen S, Jin X, Shao ZM. Value of Ki-67 expression in triplenegative breast cancer before and after neoadjuvant chemotherapy with weekly paclitaxel plus carboplatin. Sci Rep 2016; 6: 30091.
[20] Mohammed ZM, McMillan DC, Elsberger B, Going JJ, Orange C, Mallon E, Doughty JC, Edwards J. Comparison of Visual and automated assessment of Ki-67 proliferative activity and their impact on outcome in primary operable invasive ductal breast cancer. Br J Cancer 2012; 106: 383-388.
[21] Nishimura R, Osako T, Okumura Y, Tashima R, Toyozumi Y, Arima N. Changes in the ER, PgR, HER2, p53 and Ki-67 biological markers between primary and recurrent breast cancer: discordance rates and prognosis. World J Surg Oncol 2011; 9: 131.
[22] Keam B, Im SA, Lee KH, Han SW, Oh DY, Kim JH, Lee SH, Han W, Kim DW, Kim TY, Park IA, Noh DY, Heo DS, Bang YJ. Ki-67 can be used for further classification of triple negative breast cancer into two subtypes with different response and prognosis. Breast Cancer Res 2011; 13: R22.
[23] Wang X, Shi X, Li F, Liu J, Cheng C. Application of analytical and preparative high-speed counter-current chromatography for the separation of Z-ligustilide from a crude extract of angelica sinensis. Phytochem Anal 2008; 19: 193-197.
[24] Zheng YZ, Choi RC, Li J, Xie HQ, Cheung AW, Duan R, Guo AJ, Zhu JT, Chen VP, Bi CW, Zhu Y, Lau DD, Dong TT, Lau BW, Tsim KW. Ligustilide suppresses the biological properties of Danggui Buxue Tang: a Chinese herbaldecoction composed of radix astragali and radix angelica sinensis. Planta Med 2010; 76: 439-443.
[25] Wang M, Jia M. Advances in the study on chemical constituents of angelica dahurica. Zhong Yao Cai 2002; 25: 446-449.
[26] Wu Z, Uchi H, Morino-Koga S, Nakamura-Satomura A, Kita K, Shi W, Furue M. Z-Ligustilide inhibits benzo(a)pyrene-induced CYP1A1 upregulation in cultured human keratinocytes via ROS-dependent Nrf2 activation. Exp Dermatol 2014; 23: 260-265.
[27] Su YW, Chiou WF, Chao SH, Lee MH, Chen CC, Tsai YC. Ligustilide prevents LPS-induced iNOS expression in RAW 264.7 macrophages by preventing ROS production and down-regulating the MAPK, NF-κB and AP-1 signaling pathways. Int Immunopharmacol 2011; 11: 1166-1172.
[28] Qi H, Han Y, Rong J. Potential roles of PI3K/Akt and Nrf2-Keap1 pathways in regulating hormesis of Z-ligustilide in PC12 cells against oxygen and glucose deprivation. Neuropharmacology 2012; 62: 1659-1670.
[29] Xu W, Yang L, Li J. Protection against beta-amyloid-induced neurotoxicity by naturally occurring Z-ligustilide through the concurrent regulation of p38 and PI3-K/Akt pathways. Neurochem Int 2016; 100: 44-51.
[30] Zhang YT, Li FM, Guo YZ, Jiang LR, Ma J, Ke Y, Qian ZM. (Z)-ligustilide increases ferroportin1 expression and ferritin content in ischemic SH-SY5Y cells. Eur J Pharmacol 2016; 792: 48-53.
[31] Zhu MD, Zhao LX, Wang XT, Gao YJ, Zhang ZJ. Ligustilide inhibits microglia-mediated proinflammatory cytokines production and inflammatory pain. Brain Res Bull 2014; 109: 54-60.
[32] Kuang X, Du JR, Chen YS, Wang J, Wang YN. Protective effect of Z-ligustilide against amyloid beta-induced neurotoxicity isassociated with decreased pro-inflammatory markers in rat brains. Pharmacol Biochem Behav 2009; 92: 635-641.