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Abstract: Objective: To investigate the effects of thunder-fire moxibustion in rats with mammary gland hyperplasia and its related mechanisms. Method: A total of 40 non-pregnant female Sprague Dawley rats were obtained; 10 were randomly selected as the control group, and the rest were used for constructing models of mammary gland hyperplasia. The model rats were randomized into the model group, the tamoxifen group, and the moxibustion group, with 10 in each group. The control and model groups were fed normally; the tamoxifen group was intragastrically administrated with tamoxifen, and the moxibustion group received thunder-fire moxibustion therapy. The rats’ serum and their mammary gland tissues as well as their ovaries were collected after 30 days of the aforementioned treatments. The serum estradiol and progesterone levels in the rats were determined using ELISA, the morphological and structural changes of the mammary gland tissues were observed through H&E staining, and the levels of the hedgehog signaling pathway-related proteins in the ovarian tissue were determined using Western blotting. Results: The nipple diameter in the moxibustion group was significantly smaller than it was in the model group and the tamoxifen group (both P<0.05). The body weight in the moxibustion group was significantly higher than it was in the model group and the tamoxifen group (both P<0.05). In the control group, the mammary epithelial cells of the healthy rats were relatively orderly arranged, with fewer lobules and no enlargement of the acinar lumina, but in the model group, the arrangement of the ductal epithelial cells was in disorder, with more lobules and acini, as well as significant enlargement in the acinar lumina, and the features of mammary gland hyperplasia were obvious. In the tamoxifen group, the arrangement of the mammary epithelial cells was in slight disorder, with a certain increase in the number of lobules and enlargement in the acinar lumina. The tissue morphology of the rats in the moxibustion group was similar to the morphology in the control group. The serum estradiol (E2) level in the moxibustion group (56.72 ± 4.87 ng/L) was significantly lower than it was in the model group and the tamoxifen group (both P<0.05). The serum progesterone (P) level in the moxibustion group (38.27 ± 3.74 mg/L) was significantly higher than it was in the model group and the tamoxifen group (both P<0.05). The relative expression levels of PTCH1, SMO, and GLI1 in the moxibustion group (0.31 ± 0.05, 0.25 ± 0.04, 0.33 ± 0.06, respectively) were significantly higher than those in the Model group and the tamoxifen group (all P<0.05). Conclusion: Thunder-fire moxibustion therapy can effectively improve the secretion of E2 and P in rats with mammary gland hyperplasia and reverse mammary gland hyperplasia lesions, which may be related to the activation of the hedgehog signaling pathway.

Keywords: Thunder-fire moxibustion, mammary gland hyperplasia, Hedgehog signaling pathway

Introduction

Mammary gland hyperplasia is at present a clinical gynecological disease with a high incidence. Unilateral or bilateral breast swelling, often accompanied by pain and other complications, is the main manifestation of the hyperplasia. Its appearance is related to the patients' dietary habits, endocrine status, mental state, and menstrual cycle [1]. Clinical studies have shown that women's ovaries, beginning at puberty, begin to secrete sex hormones such as estradiol (E2) and progesterone (P) in a large amount, which promotes the development of ducts, acini and lobules of the mammary glands, thus inducing breast maturation; many receptor proteins of sex hormones in breast tissues are very sensitive to the changes in sex hormone levels, and an excessive secretion of the hormones can result in excessive growth of the mammary glands [2]. The hedgehog signaling pathway, originally discovered in the study
of the embryonic development of drosophila, has important regulatory effects on cell growth, maturation and other important physiological activities [3]. A dysfunction of the hedgehog signaling pathway can lead to the disorder of sex hormone secretion in the ovaries thus affecting the development of the mammary glands or causing mammary gland hyperplasia or other pathological changes. There are studies at present indicating that the incidence of mammary gland hyperplasia presents a rapid upgrowing trend, and about 70% of women have mammary gland hyperplasia of varying degrees [4]. A study found that surgical resection for mammary gland hyperplasia is effective but may cause relatively great trauma to the patients [5]. Thunder-fire moxibustion is a type of special moxibustion method reformed in moxa formula based on conventional moxibustion according to the principle of pattern differentiation and treatment of traditional Chinese medicine, and it mainly works for treating diseases through the delivery of heat, infrared rays, and medicinal factors from burning moxa sticks containing certain Chinese herbs to acupuncture points and meridians on the body or the diseased area. A study showed that thunder-fire moxibustion has a certain effect on gynecological inflammation, endometriosis, and chronic arthritis, but it is rarely used in the treatment of mammary gland hyperplasia [6]. In this study, the effect and related mechanisms of thunder-fire moxibustion on mammary gland hyperplasia in rats was studied, seeking to providing a basis for clinical treatment among patients with mammary gland hyperplasia.

Materials and methods

Animal modeling

A total of 40 female, 8-10 week old, non-pregnant SD rats of the cleanliness grade, weighing 200.41 ± 15.62 g, were purchased from the Laboratory Animal Center of Soochow University. This study was approved by the Animal Ethics Committee of the Chinese Medicine Hospital of Leshan. To establish rat models of mammary gland hyperplasia, the rats were intraperitoneally injected with estradiol benzoate (Shanghai General Pharmaceutical Co., Ltd.) at a dose of 0.5 mg/kg for 30 consecutive days, followed by an intraperitoneal injection of progesterone (Zhejiang Xianju Pharmaceutical Co., Ltd.) at a dose of 5 mg·kg⁻¹ for 5 consecutive days. The diameters of the first pair of mammary nipples of the rats were measured with a Vernier caliper, then the rats were weighed, and then part of the tissues of the second pair of breasts of the rats were removed by aseptic wedge resection. The hyperplasia of the ducts and acini in all the mammary tissues of each rat was confirmed by pathological tissue sections, suggesting that the model for mammary gland hyperplasia was successfully constructed.

Animal grouping

After we obtained the rats, the 40 animals were randomized into four groups, and 10 were randomly selected as the control group, without any treatment. The remaining 30 were used to construct models of mammary gland hyperplasia, and no animals died during the modeling. The 30 modeled rats were randomized into three groups, the model group, the tamoxifen group, and the moxibustion group, with 10 rats in each group. All recruited rats were caged individually in a climate-controlled room (24 ± 2°C and 45%-55% relative humidity) under a 12/12 hour dark/light cycle with ad libitum access to a standard rat chow diet and water. The tamoxifen group was given tamoxifen by intragastric administration at a dose of 0.002 g/kg in a suspension once a day, and tamoxifen tablets (Yangtze River Pharmaceutical Group) were crushed and ground and then made into the suspension with 2 mL of 0.5% carboxymethyl cellulose sodium. In the moxibustion group [8], the hair of the rats around the mammary gland was removed, and then the rats were fixed on the metal rat frame, followed by a suspended moxibustion right over the mammary gland part with an appropriate distance (about 2 cm) through a special suspended moxibustion box in which a thunder-fire moxa stick (purchased from Zhao’s Thunder-fire Moxibustion Traditional Medicine Research Institute, Chongqing, China) would burn after being lit up. The moxa stick contains several Chinese herbal medicinal ingredients from Chinese agalwood (Lignum Aquilariae Resinatum), pangolin scales (Squama Manis), dried ginger (Rhizoma Zingiberis), virgate wormwood herb (Herba Artemisiae Scopariae), common aucklandia root (Radix Aucklandiae), and incised notopterygium rhizome & root (Rhizoma seu Radix Notopterygii). The moxibustion for the rats lasted about 15 min, and it was adminis-
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Enzyme-linked immunosorbent assay (ELISA) for estradiol and progesterone

After anesthetization, 3 mL of blood was taken from the abdominal aorta of all the rats, and then it was stored at 4°C for 1 h. Thereafter the blood was centrifuged at 2,500 r·min⁻¹ for 15 min, and then the supernatants (i.e., serum) were collected and stored at -80°C for the subsequent ELISA for determining serum E2 and P levels. According to the ELISA kit instructions, after coating the enzyme label plate with antibodies, a gradient dilution was carried out on the samples and standards, followed by setting standard wells, reference wells and sample wells, and then the plates were kept at 37°C for 15 min after adding the supernatants obtained from the blood samples. After washing the wells, horseradish peroxidase (HRP)-labeled secondary antibody was added into each well and the plates were kept at 37°C for 15 min. After washing the wells again, the reaction substrate was added for color development, and the plates were then kept at 37°C for 10 min. A stop solution was thereafter added to stabilize the color development, and the optical density (OD) value in each well was then determined at 490 nm using a microplate reader (BioTek, USA).

Western blotting hedgehog signaling pathway related proteins

After blood collection under anesthesia, the rats were sacrificed by cervical dislocation. The ovary tissues of the rats on the right side were collected and then pulverized and ground after being immersed in liquid nitrogen. Then the total protein of the samples was extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer, and the protein concentration was determined by the bicinchoninic acid (BCA) assay. After bating the protein sample in boiling water at 100°C for 5 min, and then cooling it, 20 μg of sample protein was added into each well. The protein was separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane by the wet transfer method. After being sealed through immersing 1× TBST (a mixture of tris-buffered saline and polysorbate 20) buffer containing 5% skimmed milk powder, the membrane was rinsed with a TBST buffer three times. Then the membrane was incubated with sheep anti-rat primary antibodies against PTCH1, SMO, and Gli1 overnight at 4°C in a refrigerator. After being rinsed three times with a TBST buffer, the membranes were incubated with HRP-labeled rabbit anti-sheep IgM secondary antibodies for 1 h at room temperature. After rinsing again three times with the TBST buffer, images of the membrane proteins were developed by electrochemiluminescence (ECL) imaging, and the gray values of the target proteins were evaluated by ImageJ software (developed by the National Institutes of Health).

Hematoxylin & eosin staining for mammary gland tissues

The mammary tissues of the first pair of breasts of the rats on the right side were obtained after they were sacrificed, and then they were fixed with 4% paraformaldehyde solution, followed by routine paraffin sectioning. The paraffin sections were dewaxed in xylene for 10 min, cleaned in graded alcohol solutions, and stained with a hematoxylin solution for 5 min. After they were washed with distilled water, the sections were differentiated by a 1% hydrochloric acid alcohol solution, and then they were rinsed with distilled water until the nucleus turned blue. Then the sections were stained with an eosin solution for about 1 min, and then they were cleaned by graded alcohol solutions, followed by a transparent procedure using xylene and sealing. The sealed sections were visualized with a Leica inverted fluorescence confocal microscope (Leica, Wetzlar, Germany).

Statistical analyses

Data in this study were analyzed using the SPSS 19.0 software package (SPPS Inc., Chicago, II, U.S.A). The measurement data were expressed as the mean ± standard deviation (x ± sd). Comparisons among multiple groups were carried out using one-way analysis of variance, and the differences between two groups were compared using the Bonferroni post-hoc test. P values <0.05 were considered statistically significant.
Result

The effect of thunder-fire moxibustion on the nipple diameter and body weight of rats with mammary gland hyperplasia

The diameter of the rat nipples in moxibustion group was 1.14 ± 0.07 mm, which was significantly smaller than the rats in the model group and the rats in the tamoxifen group (both P<0.05). There was no significant difference in nipple diameter between the moxibustion and control groups (P>0.05). See Figure 1A. The weight of the rats in moxibustion group was 271.65 ± 14.62 g, which was significantly higher than the weight in the model and tamoxifen groups (both P<0.05). There was no significant difference in body weight between the moxibustion and control groups (P>0.05). See Figure 1B.

The effect of thunder-fire moxibustion on the morphology of the mammary tissues of rats with mammary gland hyperplasia

In the control group, the mammary epithelial cells of the healthy rats were relatively orderly arranged, with fewer lobules and no enlargement of the acinar lumina, but in the model group, the arrangement of the ductal epithelial cells was in disorder, with more lobules and acini, as well as significant enlargement in the acinar lumina, and the features of mammary gland hyperplasia were obvious. In the tamoxifen group, the arrangement of the mammary epithelial cells was in slight disorder, with a certain increase in the number of lobules and enlargement in the acinar lumina. The tissue morphology of the rats in the moxibustion group was similar to that in the control group. See Figure 2.

The effects of thunder-fire moxibustion on the serum E2 and P levels of rats with mammary gland hyperplasia

The serum estradiol (E2) level in the moxibustion group was 56.72 ± 4.87 ng/L, which was significantly lower than the levels in the model and tamoxifen groups (both P<0.05). There was no significant difference in serum E2 levels between the moxibustion and control groups (P>0.05). The serum progesterone (P) level in the moxibustion group was 38.27 ± 3.74 mg/L, which was significantly higher than the level in the model and tamoxifen groups (both P<0.05). There was no significant difference in serum P level between the moxibustion and control groups (P>0.05). See Figure 3.

The effect of thunder-fire moxibustion on the hedgehog signaling pathway in rats with mammary gland hyperplasia

The relative expression level of PTCH1 in the moxibustion group was 0.31 ± 0.05, which was
significantly higher than the level in the model and tamoxifen groups (both \( P < 0.05 \)), but with no significant difference between the moxibustion and control groups (\( P > 0.05 \)). The relative expression levels of SMO in the moxibustion group was 0.25 ± 0.04, which was significantly higher than the levels in the Model and tamoxifen groups (both \( P < 0.05 \)). The relative expression level of GLI1 in the moxibustion group was 0.33 ± 0.06, which was significantly higher than the level in the model and tamoxifen groups (both \( P < 0.05 \)). There was no significant difference in the relative expression level of GLI1 between the moxibustion and control groups (\( P > 0.05 \)). See Figure 4.

Discussion

Mammary gland hyperplasia is a common gynecological disease, with a relatively high incidence among young and middle-aged women, and an abnormal structure of the mammary glands is the main manifestation [9]. Epidemiological studies have found that dietary habits, mental state, drug abuse, and other factors can lead to an abnormal secretion of sex hormones in some women, which adds to the risk of mammary gland hyperplasia [10]. The relatively complex pathogenesis of mammary gland hyperplasia is related to a disorder of sexual hormone secretions in the patients.
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Anti-hormone drugs and surgical resection with certain therapeutic effects are currently the main choices for mammary gland hyperplasia in the clinic. However, hormone therapy may cause adverse reactions in the patients, and surgery presents a relatively big trauma with possible scars in the patients [11, 12]. A study found that patients with mammary gland hyperplasia treated with tamoxifen have gastrointestinal discomfort, weight gain and irregular menstruation, and the hyperplasia tends to recur when stopping the drug treatment [13]. Therefore, it is of great clinical value to explore the therapeutic effect of thunder-fire moxibustion on mammary gland hyperplasia.

This study found that the nipple diameter of the rats with mammary gland hyperplasia after treatment with thunder-fire moxibustion was significantly reduced, and the body weight was clearly increased, compared with the model group. H&E staining showed that the ductal epithelial cells were arranged relatively orderly, with significant induction in the numbers of lobules and acini, as well as a significant enlargement in the acinar lumina, indicating that thunder-fire moxibustion can in some degree reverse the condition of mammary gland hyperplasia in rats, which may have value as a potential clinical application. E2 and P play crucial regulatory roles in the development of mammary tissues; an increased E2 level promotes the expansion of mammary ducts and lobules; P can maintain the normal development of breast acini and antagonize the physiological function of E2, thus inhibiting the sensitivity of mammary tissues to E2 [14-16]. In this study, it was found by ELISA that serum E2 levels increased and P levels decreased in rats with mammary gland hyperplasia, and serum E2 decreased and P levels increased in rats with mammary gland hyperplasia after thunder-fire moxibustion therapy, suggesting that thunder-fire moxibustion can significantly regulate the secretion of serum E2 and P in rats with mammary gland hyperplasia. Consistent with this study, a study found that rats with endometriosis and treated with thunder-fire moxibustion can effectively inhibit the secretion disorders of serum E2 and P [17]. Patch 1, SMO, and Gli1 are important functional proteins of the hedgehog signaling pathway mainly involving in cell proliferation, differentiation, and growth [18-20]. Studies have found that the down-regulation of Patch 1 expression in ovarian granulose cells can result in an abnormal expression of SMO and Gli1 genes, subsequently with the abnormal function of the hedgehog signaling pathway, thus leading to a secretion disorder of the ovarian sex hormone [21]. In this study, the relative expression levels of Patch 1, SMO, and Gli1 in the moxibustion group were significantly higher than those in the model and tamoxifen groups, suggesting that thunder-fire moxibustion can stimulate the activity of the hedgehog signaling pathway in the ovary. During thunder-fire moxibustion therapy, complex active ingre-
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Gradients of traditional Chinese medicine and physical stimulation work together on mammary gland hyperplasia in rats. Therefore, the mechanism of thunder-fire moxibustion in the treatment of mammary gland hyperplasia is complex. This study only found the effect of thunder-fire moxibustion on the E2 and P levels in rats as well as the hedgehog signaling pathway. Hence, it is necessary to further analyze the treatment mechanisms of thunder-fire moxibustion in a more refined way.

In summary, thunder-fire moxibustion therapy can effectively improve the secretions of E2 and P in rats with mammary gland hyperplasia and reverse the pathological changes of mammary gland hyperplasia, which may be related to the activation of the hedgehog signaling pathway.

Disclosure of conflict of interest

None.

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