Effect of Xihuang Pill on microcirculation in DMBA combined estrogen and progesterone induced breast precancerous lesions rats

Dehui Li*, Yifan Su, Huanfang Fan, Chunxia Sun, Changhui Han, Ma Pan, Jiaojiao Yan
Affiliated Hospital of Hebei University of TCM, Hebei Provincial Hospital of TCM, Shi Jiazhuang 050011, China
*Corresponding author’s e-mail: 258289951@qq.com

Abstract. Objective: To observe the effect of Xihuang Pill on microcirculation in 7,12-dimethylbenzoanthracene (DMBA) combined estrogen and progesterone induced breast precancerous lesions rats. Method: DMBA combined estrogen and progesterone were used to establish the model of breast precancerous lesions for 10 weeks in SD rats, then the low, medium and high dose Xihuang Pill (0.27, 0.55, 1.37g·kg$^{-1}$) was given intragastric administration, and tamoxifen (4 mg·kg$^{-1}$) was given intragastric administration as a positive control after the breast precancerous lesions models established successfully. The breast microcirculation was measured by laser Doppler flowmetry (LDF) in rats after 4 weeks of Xihuang Pill and tamoxifen intragastric administration. Result: Compared with the normal control group, the microcirculation perfusion of breast in the disease model group decreased significantly ($P<0.05$). Compared with the disease model group, the breast microcirculation perfusion in each dose group of Xihuang Pill increased significantly ($P<0.05$). Conclusion: The mechanism of Xihuang Pill in the treatment of breast precancerous lesions may be related to the improvement of breast microcirculation.

1. Introduction
The incidence and mortality of breast cancer is the highest in the world's female cancer, which seriously threatens women's health and life safety [1]. Modern research has confirmed that the occurrence of breast cancer has experienced a continuous pathological process of "normal epithelium→non-atypical hyperplasia→atypical hyperplasia→in situ carcinoma→invasive carcinoma". In the development of normal epithelium to becoming invasive carcinomas, atypical hyperplasia and in situ carcinoma may be in a relatively stable state for a long time, which is called precancerous lesions of breast cancer. As an important stage of the occurrence and development of breast cancer, precancerous lesions can be identified and active intervention as early as possible, which can block or even reverse the canceration process and effectively reduce the incidence and mortality of breast cancer [2]. At present, the key of prevention and treatment of breast cancer is to intervene breast precancerous lesions and block their development to breast cancer. Modern research shows that the blood viscosity of patients and the detection rate of blood flow signal increases with increasing of the hyperplastic degree of mammary epithelium [3,4]. According to traditional chinese medicine, "phlegm toxin and blood stasis" is the core pathogenesis of the occurrence and development of breast precancerous lesions [5, 6]. The classic prescription Xihuang pill (XHP) has the effects of clearing heat and detoxicating, reducing phlegm and resolving masses, promoting blood circulation and
removing blood stasis, decreasing swelling and relieving pain. It has been used in clinical practice to treat breast precancerous lesions and breast cancer with remarkable effect [7, 8]. In our previous study [9], we found that Xihuang pill extract can inhibit the cell viability and induce the apoptosis of breast precancerous cells in vitro via inhibition of the expression of mTOR and VEGF in PI3K/Akt/mTOR signaling pathway. In this study, we observed the effect of Xihuang Pill on microcirculation in DMBA combined estrogen and progesterone induced breast precancerous lesions rats, and explored the mechanism of intervention of Xihuang Pill on precancerous lesions of breast.

2. Laboratory Animals and Materials

2.1 Experimental Animal.
Sixty SPF-grade female SD rats with body weight of (180 ± 20) g, provided by Hebei experimental animal center, quality license: 1705351. The experiment began after one week adaptive feeding.

2.2 Experimental Drugs.
Estradiol benzoate injection, produced by Ningbo No.2 hormone factory (batch No.: 110252511); progesterone injection, produced by Ningbo No.2 hormone factory (batch No.: 110251670); tamoxifen citrate tablets, Yangzijiang Pharmaceutical Group Co., Ltd., (batch No.: 17041311); 7, 12-dimethylbenz[a]anthracen (DMBA), TCI, (cas57-97-6); Xihuang pill, Zhejiang Tianyitang Pharmaceutical Co., Ltd Division, (batch No: 1703011).

3. Experimental Method

3.1 Animal Model Establishment[10].
On the first day of the experiment, a single dose of DMBA in sesam oil (7 mg·mL⁻¹) was given to rats (1 mL·100g⁻¹) by gavage. Then 5 days for 1 cycle, On day 1 to 3, Estradiol benzoate injection was injected intramuscularly into the hind leg (0.5 mg/kg) of rats, on the 4th day, injected Progesterone injection (4 mg/kg), on the 5th day only observation. The rat model of breast precancerous lesions was established in 12 cycles.

3.2 Grouping and Administration Method.
60 rats were randomly divided into normal control group (10 rats) and disease model group (50 rats). Normal control group: after one-time gavage of 1 mL·100g⁻¹ sesam oil without DMBA, the rats were fed regularly. Disease model group: establish model according to the above modeling method, the rats were fed regularly. After model establishment, the rats of model group were randomly divided into five groups with 10 rats in each group: disease model group, tamoxifen group, XHP low-dose group, XHP middle-dose group and XHP high-dose group. Disease model group: regular feeding 4 weeks. Tamoxifen group: rats were administered with tamoxifen (4 mg·kg⁻¹) by gavage once a day for 4 weeks. XHP group rats were given XHP (0.27, 0.55, 1.37 g·kg⁻¹) by gavage once a day for 4 weeks.

3.3 Hemorheology Test.
All rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 mL·100g⁻¹) after 4 weeks since intragastric administration, and were put into incubator for 10min. Then the perfusion of the second pair of mammary microcirculation was measured by laser Doppler microcirculation analyzer.

3.4 Statistical Methods.
SPSS 20.0 statistical software was used to analyze the data. Data is expressed as mean±standard deviation (X ± s). One way ANOVA analysis was used for comparison between groups, P values <0.05 were considered significant.
4. Result

4.1 Rats weight change
There was no statistical difference in the weight of each group before the experiment. The weight of each group increased in the 10th week of the experiment compared with that before the experiment, and there was no statistical difference in each group in the 10th week. The weight of each group increased in the 14th week of the experiment compared with that in the 10th week, and the weight of rats in tamoxifen group was significantly lower than that of other groups in the 14th week (P < 0.05). (see Table 1).

| group                  | dose (mg·kg⁻¹) | 0 week       | 10 week      | 14 week      |
|------------------------|----------------|--------------|--------------|--------------|
| Normal control group   | -              | 177.21±15.38 | 209.00±30.66 | 259.14±19.53 |
| Disease model group    | -              | 184.50±19.00 | 214.00±18.74 | 238.50±24.01 |
| Tamoxifen group        | 4              | 181.75±20.11 | 200.83±17.29 | 214.30±15.99*|
| XHP low-dose group     | 270            | 188.25±20.10 | 210.50±18.83 | 234.90±26.20 |
| XHP middle-dose group  | 550            | 185.92±13.23 | 220.92±17.18 | 243.90±20.80 |
| XHP high-dose group    | 1370           | 183.67±16.74 | 211.17±19.75 | 236.40±22.53 |

Note: comparison with normal control group,*P<0.05.

4.2 Pathological Changes of Rat Breast.
The breast tissue of each group was stained with HE and observed pathomorphological changes under light microscope. The results showed that the breast tissue of the normal control group was normal gland; the breast tissue of the disease model group was mainly atypical hyperplasia of grade II-III, with the presence of intraductal carcinoma of breast gland; General hyperplasia was the main form in each dose group of Xihuang Pill, with the presence of atypical hyperplasia of type I, indicating that Xihuang pill could block and reverse the histopathological changes of breast tissue induced by DMBA combined with estrogen and progestogen.

4.3 Effect of Xihuang Pill on microcirculation perfusion of rat breast.
The results showed that the microcirculation perfusion in the disease model group was significantly lower than that in the normal control group (P < 0.05), there was no statistical significance between the disease model group and tamoxifen group. Compared with the disease model group, the microcirculation perfusion increased significantly with increasing doses of XHP (P < 0.05), (see Table 2).

| group               | dose (mg·kg⁻¹) | microcirculation perfusion |
|---------------------|----------------|---------------------------|
| Normal control group| -              | 119.29±18.36              |
| Disease model group | -              | 83.20±18.49*              |
| Tamoxifen group     | 4              | 78.40±16.99               |
| XHP low-dose group  | 270            | 90.40±22.19*              |
| XHP middle-dose group| 550           | 97.90±16.83*              |
| XHP high-dose group | 1370           | 112.2±24.37*              |

Note: comparison with the control group,*P<0.05;Comparison with disease model group, P<0.05.
5. Discuss
The breast microcirculation perfusion can directly reflect the local blood flow situation. In this study, DMBA combined with estrogen and progestogen was used to induce the rat model of breast precancerous lesions. The detection results showed that the breast microcirculation perfusion in the disease model group was significantly lower than that in the normal control group, indicating that breast microcirculation disturbance exist in model rats. Our previous research confirmed that the patients with breast precancerous lesions generally have high blood viscosity, coagulation and aggregation, the abnormal hemorheology can directly affect the blood perfusion of the breast, increase the resistance of blood flow, if it blocked in the breast collaterals and form lumps, which will promote mammary gland hyperplasia and breast cancer carcinogenesis [4]. This study proves that breast precancerous lesions rats exist hypercoagulable state.

Xihuang pill was created by Wang Hongxu, a famous doctor in the Qing Dynasty. It is recorded in waike zhengzhi quansheng ji and composed of bezoar, musk, frankincense and myrrh. It has excellent effectiveness in the treatment of breast cancer and precancerous lesions. In this study, Xihuang pill was used to treat the precancerous lesions of the rat, pathomorphology shows that the breast tissue in the disease model group was mainly of atypical hyperplasia grade II-III, and that in each dose group of Xihuang pill was mainly of non-atypical hyperplasia, which indicated that the therapeutic effect of Xihuang pill was very good in the stage of precancerous lesions developing to breast cancer. To a certain extent, Xihuang Pill can block and reverse histopathological changes of breast in rats with precancerous lesions induced by DMBA combined with estrogen and progestogen. This study showed that breast microcirculation perfusion in low, middle and high dose Xihuang pill group was significantly improved compared with that in disease model group, which indicated that Xihuang pill could improve the blood flow of breast in precancerous lesion model rats, promote the elimination of local metabolites, and remove blood stasis, it is vital for effective prevention and therapy of breast cancer.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (Grants No. 81603412); Key r&d Projects of Hebei Province (Grants No. 18277731D); Scientific Research Project of Hebei Administration of Traditional Chinese Medicine (Grants No. 2017163); General Projects for Improving Scientific Research Capacity of Hebei College of Traditional Chinese Medicine (Grants No. KTY2019009); Hebei Key Laboratory of Chinese Medicine Research on Cardio-Cerebrovascular Disease; Key Laboratory of Integrated Traditional Chinese and Western Medicine Hepatonephrosis in Hebei Province (Grants No. A201902).

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