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The anti-hyperplasia of mammary gland effect of protein extract HSS from *Tegillarca granosa*

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ABSTRACT

Tegillarca granosa Linnaeus, possesses various biological functions and has been used a Chinese traditional medicine more than one century, but there is no report about anti-hyperplasia of mammary gland (HMG) activity of drugs from *T. granosa*. In this study, we investigated the anti-HMG effect of protein extract named HSS from *T. granosa*. The HMG model of virgin female Sprague Dawley rats was prepared by injecting estrogen in the thigh muscle of the rats and progestogen consecutively. HMG rats were treated with either HSS or positive control drug by i.g. for 35 consecutive days. In order to evaluate anti-HMG activity of HSS, Changes of nipple height and diameter, serum sex hormones levels, organ indexes and pathologic changes of mammary gland were performed. Body weight, food intake, pathomorphology examination of organs (heart, liver, spleen, lung, kidney), hematological and biochemical analysis were performed to evaluate the toxicity of HSS. HSS could significantly reduce nipples height and diameter, increase P concentration of HMG rat serum, spleen and thymus index, decrease uterus index, and has therapeutic effect on rat HMG and no toxicity at 500 mg/kg/day. The anti-HMG mechanism of HSS may be related to AP-2α and P53. HSS has protective and therapeutic effects on HMG rats, and may be a promising agent for treating hyperplasia of mammary glands.

1. Introduction

Hyperplasia of mammary gland (HMG) is a common disease in the middle-aged women, which is a kind of pathological hyperplasia of lobules of mammary gland induced by the balance disorder of estrogen and progesterone [1]. The morbidity of HMG is increasing nowadays, with a risk of causing mammary carcinoma [2]. So it is important for human health to discovery more convenient and effective new drugs with few side effects for treating hyperplasia of mammary glands and to explore the anti-HMG mechanisms of these drugs for blocking its development to breast cancer.

Marine drugs from ocean organisms (such as *Tegillarca granosa* Linnaeus), have various bioactivities. Previous reports indicated that active protein components from *T. granosa* have antibacterial immunity [3,4], antitumor activities [5–7], but there is no report about anti-HMG activity of drugs from *T. granosa*.

T. granosa, belongs to Arcidae family, is widely distributed in mainland China and used a Chinese traditional medicine more than one century [8]. Activity protein components from *T. granosa* can inhibit the proliferation of tumor cells [9], and have established the quality control methods and HPLC fingerprint [10], and the extraction method of activity protein components have been obtained China patent [11]. Activity protein named HSS is a protein extracts with molecular weights of 23 KDa from *T. granosa* [10,11]. HSS can inhibit the growth of transplanted k562 tumor, drug-resistant K562/ADM tumor [12], renal metastatic tumor [13], SPC-A-1 xenograft [14] and induce tumor cells apoptosis in renal tumor OS-RC-2 cells [13], ovarian cancer OVCAR-3 cells [15], k562 cells [16], K562/ADM cells [17,18], human hepatocellular carcinoma BEL-7402 cells [19]. HSS also plays anti-proliferative activity [15,16] and immunomodulation by increasing the number of phagocytizing macrophages and neutrophils in mice with Ehrlich

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ascites tumor [20,21], and increase remission rate in patients with advanced renal cell cancer [22], and is a potential adjuvant chemotherapy for NSCLC treatment [14].

In the present study, we evaluated the anti-HMG effects of HSS in HMG rats which were induced by estrogen and progestogen. In order to evaluate the toxicity of HSS, body weight, food intake, pathomorphology examination of organs (heart, liver, spleen lung, kidney), hematological and biochemical analysis were performed. This is first study on anti-HMG effect of HSS *in vivo*.

2. Materials and methods

2.1. Chemicals

HSS was provided by Qindao Marine Cancer Hospital Co., Ltd. The detailed method of protein extraction from *T. granosa*. was provided by Chen (2006) [10].

Tamoxifen Citrate Tablet (TMXF) was another reference drug (positive control) which has been used to treat HMG and obtained from Yangtze River Pharmaceutical (Group) Co., Ltd. Estrogen benzoate injection was purchased from Ningbo Second Hormone Factory. Progestogen injection was purchased from Shanghai General Pharmaceutical Co., Ltd. Progesterone (P), estradiol (E2), prolactin (PRL) and testosterone (T) ELISA kits were purchased from ShangHai HengYuan Biological Technology Co., Ltd. All other regents and solvents were of analytical grade.

2.2. Animals and treatments

Virgin female Sprague Dawley weighing 180–220 g were supplied by Vital River Laboratory Animal Technology Co. Ltd. Experimental protocols (No. AMP-310) followed standards and policies of Shandong Academy of Pharmaceutical Sciences Animal Care and Use Committee. The rats were housed in plastic cages with room temperature of $24 \pm 1\,^{\circ}C$ with a relative humidity of $55 \pm 5\%$ under a 12 h light-dark cycle, and provided with rodent chow and water *ad libitum*. They were allowed for acclimation for a week before use.

2.3. Animal model and experimental groups

The rats were randomly divided into six groups (n = 10), rats in the normal control group were administered with normal saline intramuscularly, rats in other groups were treated with estrogen (0.5 mg/kg) intramuscularly for 30 days, and followed with progestogen (5 mg/kg) for another 5 days to induce HMG model (Rao et al., 1992; Milliken et al., 2002). From the 36th day the rats in the normal control group and HMG model group were received normal saline by gavage. The rats in TMXF groups were treated with TMXF (2 mg/kg), and the rats of HSS group was administered with 500 mg/kg dose for 35 days.

2.4. Body weight, food intake, nipple height and diameter, and organ indexes

Body weight, food consumption and nipple height and diameter of HMG rats were recorded on the first day of administration, and then once a week thereafter and until termination.

Organ indexes (uterus, thymus and spleen) were calculated as uterus, spleen or thymus weight divided by body weight. Organ index was calculated by the following formulae:

$$\text{Organ index} = (W_{organ} \times 10)/W_{body}$$

W_{organ} and W_{body} stand for the average weights of uterus/spleen/thymus and body of the rats.

2.5. ELISA

At the end of experiments, the levels of sex hormone were observed. The rats were sacrificed and blood serum was obtained to centrifuge blood samples at 3000 rpm for 10 min at $4\,^{\circ}C$. E2, T, PRL and P concentrations in the HMG rat serum were measured. All measurements were used by enzyme-linked immunosorbent assay (ELISA) kits according to the procedures recommended by the manufacturer (ShangHai Heng Yuan Biological Technology Co., Ltd, China).

2.6. Hematological assay and serum biochemistry

At the end of the administration, blood samples were collected for analysis of hematology and serum biochemistry. Hematological parameters included white blood cell count (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelet count (PLT), lymphocytes (LYM), monocytes (MON), neutrophilic granulocytes (NEUT), eosinophils (EOS), basophilic granulocyte (BAS). The clinical chemistry parameters included alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), triglyceride (TG), total protein (TP), creatine kinase (CK), total bilirubin (TBIL), albumin (ALB) and creatinine (CREA).

2.7. Histological observations

Tissues of mammary gland from all groups were fixed in 10% buffered formalin, embedded in paraffin, sectioned into 4 μm pieces and stained with Haematoxylin-Eosin (H&E) and examined using optical microscopy.

Tissues of heart, liver, spleen, kidney, lung from HSS-high groups (500 mg/kg/day) and HMG model groups were fixed in 10% buffered formalin, embedded in paraffin, sectioned into 4 μm pieces and stained with Haematoxylin-Eosin (H&E) and examined using optical microscopy.

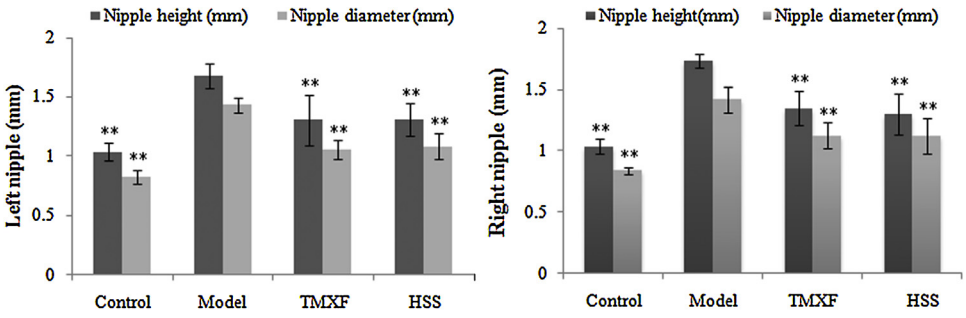

Fig. 1. Height and diameter of left and right nipples in different groups. * $P < 0.05$, ** $P < 0.01$ vs. HMG model group.

2.8. Western blot

Tissues of mammary gland were homogenized to immunoblot analysis. Protein content was determined by bradford assay. 25 μg of total protein per sample were resolved by 10% SDS-PAGE and transferred to Poluvinglidene Fluoride (PVDF) membrane (Millipore, USA). AP-2α (1:1000, Cell Signaling Technology, USA), P53 (1:1000, Cell Signaling Technology, USA) antibodies were added and treated for 1 h at room temperature and then detected the corresponding HRP-labelled secondary antiserum (1:4000, Santa Cruz Biotechnology, INC). A β-actin (1:5000, Santa Cruz Biotechnology, INC) antibody was used for loading control.

2.9. Statistical analysis

All values were expressed as the means ± SD. Data were analyzed by Student's two-tailed *t*-test. Significant difference is indicated as * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Effect of HSS on nipple height and diameter in rats

After the intragastric administration for 35d, the height and diameter of the second pairs of nipples were determined (Fig. 1). The height and diameter of nipples (Left 2 and Right 2) of HMG model rats were obviously increased by estrogen and progestogen treatment compared with normal control group ($P < 0.01$). The height and diameter of nipples (Left 2 and Right 2) of HSS group, TMXF group were reduced remarkably compared with HMG model group ($P < 0.01$). The height and diameter of nipples (Left 2 and Right 2) were no significant difference between HSS group and TMXF group.

3.2Effects of HSS on uterus, thymus and spleen index in rats

Compared with normal control group, uterus index in HMG model rats was significantly increased ($P < 0.01$), and spleen index was significantly decreased ($P < 0.01$), thymus index was also decreased (Fig. 2). These results indicate that high level of estrogen caused the increase of uterus index and the decrease of spleen and thymus index.

Uterus index of HSS group was significantly decreased ($P < 0.01$) compared with HMG model group (Fig. 2). These results may be that HSS plays anti-HMG function by decreasing uterus index to inhibit hypertrophy and hyperplasia of the uterus.

Spleen index and thymus index of HSS three groups were increased; spleen index and thymus index of HSS group had significant difference ($P < 0.05$) compared with HMG model group (Fig. 2) . These results indicated that HSS could play anti-HMG function by increasing spleen and thymus index to improve immunological function of HMG rats.

3.3. Effect of HSS on serum sex hormone levels in HMG model rats

Compared with normal control group, as shown in Fig. 3, HMG disease model rats serum progesterone (P) and testosterone (T) concentration was significantly decreased ($P < 0.05$); prolactin (PRL) was decreased; estradiol (E2) concentration was obviously increased ($P < 0.01$). Compared with HMG model group, P concentration of HSS group was obviously increased ($P < 0.01$). P concentration of TMXF group had no significant difference; E2 concentration of HSS group and TMXF group were decreased and had no significant difference ($P > 0.05$). Prolactin (PRL) concentration of HSS group and TMXF group were increased and had no significant difference ($P > 0.05$). T concentration of HSS group and TMXF group were increased and had no significant difference ($P > 0.05$) (Fig. 3). These results indicated that HSS could adjust the serum P level of HMG rats to play anti-HMG function.

3.4. Effect of HSS on mammary gland tissue pathomorphology examination in HMG model rats

Pathomorphology examination of HMG in normal control group showed no proliferative lesions, less acinars and lobules, no mammary ducts ectasia, no expansion of mammary lumens (Fig. 4A). The rats mammary of HMG model group had histological abnormalities, including significantly proliferative lesions, apparently hyperplasy, mammary ducts ectasia expansion of mammary lumens; acinars and lobules significantly increased (Fig. 4B). The degree of HMG in TMXF group was obviously alleviated, proliferative degree of mammary lobules and the number of acinars markedly decreased (Fig. 4C). The mammary gland of groups given 500 mg/kg HSS recovered well, lobule volumes and numbers of mammary lobules and acinars decreased in different degree. (Fig. 4D). These results indicated that HSS had therapeutic effect on HMG rats induced by estrogen and progestogen.

3.5. Toxicity study

The toxicity data of body weight, food intake, histopathological analysis, hematological and biochemical analysis were performed on rats of HSS group (500 mg/kg/day) and HMG model group. The body weights and food intakes of the HSS-treated HMG rats (500 mg/kg) had an alteration but no significant difference compared with HMG model rats (Fig. 5). The results of histopathologic analysis indicated that the five organs had no obvious morphologic difference between HSS-treated HMG rats (500 mg/kg) and HMG model rats (Fig. 6). There was no effect of HSS on hematological parameters of HMG rats, including WBC, RBC, HGB, HCT, MCV,MCH, PLT, LYM, MON, NEUT, EOS, BAS, and no

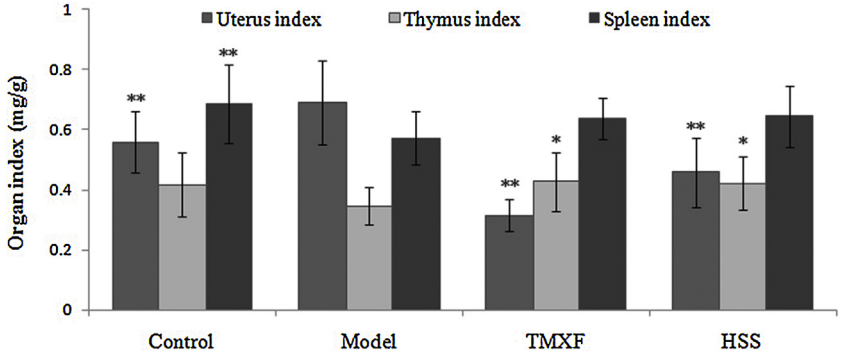

Fig. 2. Effects on uterus, thymus and spleen ovary index in different groups. * $P < 05$, ** $P < 0.01$ vs. HMG model group.

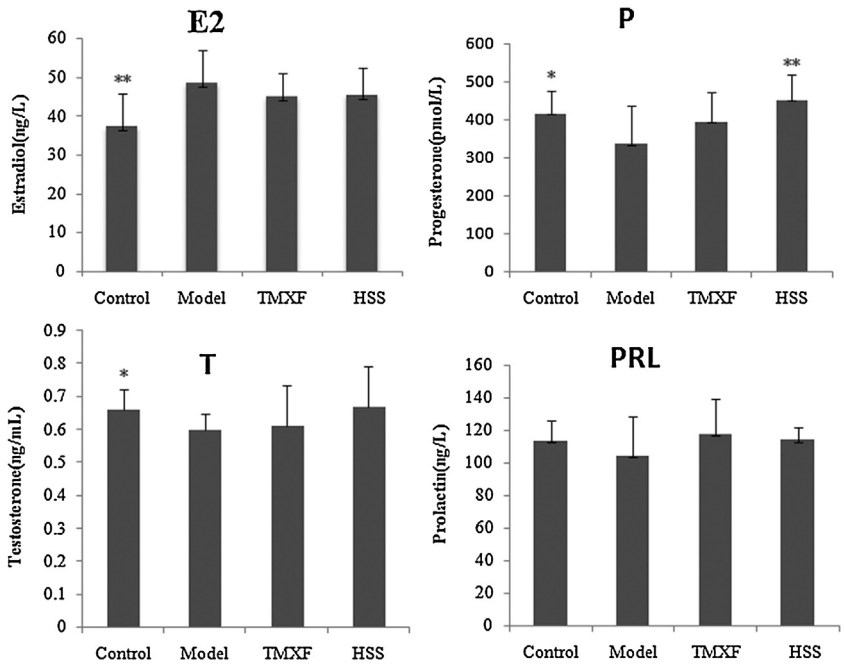

Fig. 3. Serum sex hormone concentration in different groups. * $P < 0.05$, ** $P < 0.01$ vs. HMG model group.

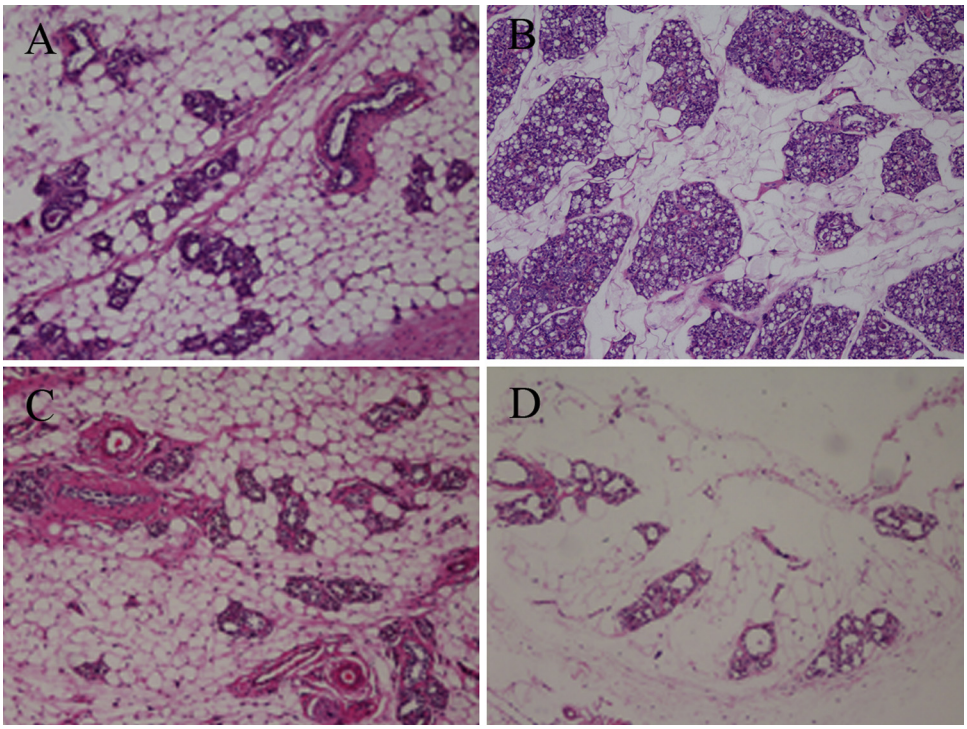

Fig. 4. Histological image of mammary gland tissue (original magnification, 100×). (A) normal control group; (B) HMG model group; (C) TMXF group (D) HSS group.

signifcent differences compared with HMG model rats ($P > 0.05$) (data not shown). No significant differences were detected between the serum biochemical parameters of HSS-treated HMG rats and HMG model rats; Average ALT, AST, ALP, ALB, BUN, TG, TP, CK, TBIL and CREA levels showed no differences between HSS group and HMG model group ($P > 0.05$)(data not shown). These results indicated that HSS has no toxic effect for HMG rats at dose of 500 mg/kg/day.

3.6. Western blot

The expression of AP-2α and P53 in mammary gland of HSS group, TMXF group and HMG model group rats were analyzed by western blot. AP-2α expression in mammary gland was significantly increased in HSS-treated HMG rats, while P53 expression was decreased in mammary gland of HSS-treated HMG rats. The

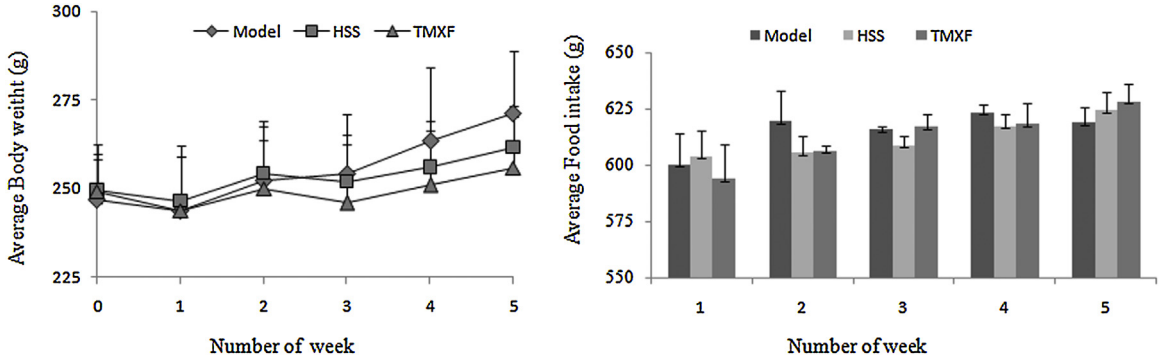

Fig. 5. Effects of HSS on the body weights and food intakes of HMG rats. Values of HSS-treated group, TMXF-treated group and HMG model group are expressed as mean ± SD (n = 10). * $P < 0.05$, ** $P < 0.01$.

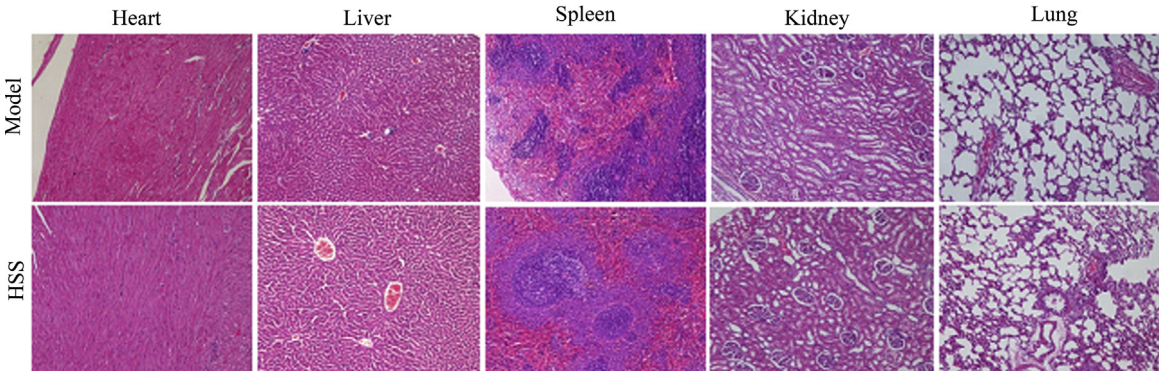

Fig. 6. Histopathologic analysis by H&E staining of organs of HSS-treated HMG rats and HMG model rats. HMG rats were treated with 500 mg/kg HSS by i.g. After 5 weeks, the rats were sacrificed and organs (heart, liver, spleen, kidney, lung) were excised. The organs were fixed by paraformaldehyde and stained by H&E to histopathologic analysis (magnification, 100 ×, Nikon, Tokyo, Japan).

expression of AP-2α and P53 in mammary gland of TMXF- treated HMG rats was similar to the expression of HSS (Fig. 7).

4. Discussion

This paper provides evidence that HSS acts as hormone-regulating agent in HMG model rats and has protective and therapeutic effects on HMG rats by adjusting endocrine system disorders and immune system function.

Thymus and spleen are the central organ of the immune system and important endocrine organ; uterus and mammary gland are the main target organ of estrogen; If exogenous estrogen levels are too high, the uterus index will be obviously increased, and thymus or spleen index will be significantly reduced [23]. The results of organ indexes (thymus, spleen and uterus) indicated that we successfully established rat model of HMG.

HSS could increase thymus or spleen index, decrease uterus index and hyperplasia of mammary gland of HMG rats, so HSS should have protective effect on thymus, spleen, uterus and mammary gland of HMG rats, and may be improve immunological function to play anti-HMG function. The anti-HMG effect mechanism of HSS on HMG rats may be by adjusting immune system function to play therapeutic effects.

Sex hormones have an important effect on the immune system, and regulate lymphoid tissue growth and development in life. The thymus is the main target organ of sex hormones on the immune system. The spleen is a place for the residence and proliferation of lymphocytes and provides specific cell immunity and humoral immune [24]. HSS could decrease P concentration and increase PRL or T concentration, so HSS may be act as a hormone-regulating agent in HMG rats and play the role of anti-HMG by regulating the immune system and hormone level.

Toxicity results indicate that HSS has no toxic effect for HMG rats at dose of 500 mg/kg/day for 35 days. The mammary gland of group given 500 mg/kg/day HSS also recovered well, the heights and diameters of nipples, lobule volumes, numbers of mammary lobules and acinars remarkably decreased in different degree. The results reveal that HSS has therapeutic effect and no toxicity effect on HMG rats induced by estrogen and progestogen.

AP-2 and P53 family memebers have different function at various developmental and lifetime periods. P53 family is related to the function and expression of AP-2 family memebers during different stages of normal development or disease processes [25]. Decreased AP-2 expression can increase P53 protein and over-

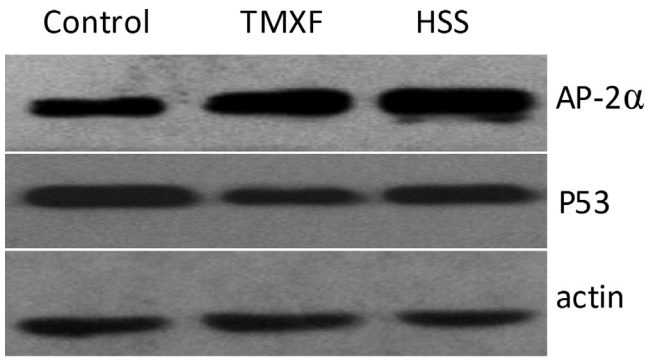

Fig. 7. Western blot analysis of mammary gland tissues. The expression of AP-2α and P53 was analyzed by Western blot in mammary gland tissues of HSS-treated, TMXF-treated and HMG model rats.

expression of AP-2α can reduce P53 expression [25,26]. AP-2α is a member of AP-2 family and required for normal growth and morphogenesis and an important for cellular functions such as proliferation, apoptosis and differentiation in the mammary epithelium under physiological conditions [27,28]. So we detected the expression of AP-2α and P53 in mammary gland. The western blot results indicate that the degree of HMG reduced by HSS may be related to the expression of AP-2α and P53 in mammary gland. The reasons may be that Over-expression of AP-2α and low-expression of P53 caused by HSS in mammary gland reduce cell proliferation, increase cell death, or lead to cell cycle arrest and then suppressed mammary gland growth and morphogenesis. So our further studies would focus on the exact anti-HMG effect mechanism of HSS.

In conclusion, HSS has protective and therapeutic effect, no toxic effect on HMG rats, and may be a new drug with low toxicity for treating hyperplasia of mammary glands and a promising agent for human mammary cancer prevention. This study is the first report of HSS therapeutic effect on HMG.

Conflict of interest statement

There is no conflict of interest in this study.

Acknowledgments

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